THE JOURNAL OF PHYSIOLOGY

EDITED FOR

THE PHYSIOLOGICAL SOCIETY

BY

Volume 108

1949

LONDON

CAMBRIDGE UNIVERSITY PRESS

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press
(Cambridge, and Bentley House, London)
Agents for Canada and India: Macmillan

CONTENTS OF VOL. 108

No. 1. 1 *March* 1949

No. 2. 15 *March* 1949

LIST OF AUTHORS

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

26 *June* 1948

23–24 *July* 1948

17–18 *December* 1948

LIST OF AUTHORS

J. Physiol. (1949) 108, 1–8 577.174.5:612.89

THE EFFECT OF ADRENOCHROME ON SYMPATHETIC NERVE STIMULATION

By G. DEROUAUX and J. ROSKAM

From the Research Laboratories of the Medical Clinic A, University of Liége, Belgium

(*Received* 14 *January* 1946)

Experiments on spontaneous haemostasis in the rabbit's ear have shown that adrenochrome, which has no vasoconstrictor action of its own (Bacq & Derouaux, unpublished), is, nevertheless, more powerful in shortening the bleeding time than adrenaline (Derouaux, 1941 *b*). This could be explained if we assumed that adrenochrome is necessary for the activity of the sympathetic and is the precursor of the transmitter substance of adrenergic nerves. The effect of adrenochrome on the bleeding time could then be explained as an indirect effect on the sympathetic nerve endings (Roskam & Derouaux, 1944).

In fact, Bacq (1933) has shown that adrenochrome restores the effect of accelerans stimulation on the perfused frog heart after prolonged perfusion, when nerve stimulation has become ineffective. No such experiments have yet been performed on warm-blooded animals, but some observations suggest that the effect of adrenaline on sympathetic stimulation may possibly be due to an action of adrenochrome. It has been shown that, after removal of the suprarenals, sympathetic stimulation quickly becomes ineffective (Elliott, 1904), and can then be restored by administration of adrenaline (Burn, 1932; Coombs, 1925; Secker, 1938), or of complete cortico-suprarenal extract (Secker, 1938; Armstrong, Cleghorn, Fowler & McVicar, 1939).

If we assume that the adrenaline injected into the circulation, or released from the suprarenals, is converted in the body to adrenochrome, these observations might suggest that adrenochrome is the precursor of the transmitter substance of adrenergic nerves. We have therefore looked for a possible reactivating effect of adrenochrome on sympathetic activity in the mammal.

METHODS

We perfused rabbits' ears with Locke's solution without interrupting their nerve supply, according to Pissemski's method (1914).

Rabbits weighing 2–2·5 kg., with large ears, were used without anaesthesia.

After isolating the right cervical sympathetic chain, we dissected a short part of the auricular artery on the same side at the base of the ear. The artery was tied with a thread and a very thin cannula was introduced into its peripheral end; the veins were cut at the base of the ear to ensure an easy flow of the perfusion fluid. A Dale-Schuster pump was used to supply the perfusion fluid.

As a rule, the perfusion flow was rather poor at the beginning, but, after 5–10 min., the arteries pulsated regularly and, in most instances, clear perfusing solution dropped from the cut veins.

The drops from the veins were registered automatically on a kymograph with a balance rheograph and an electric signal.

Stimulation of the right sympathetic chain was effected with a transformer, reducing the mains voltage to 3 V., or with an induction coil with a 4 V. accumulator in the primary.

In order to prepare the solution of adrenochrome we added 10 drops of a highly active preparation of the catechol oxidase of the mushroom *Agaricus campestris* to a Locke's solution of pH 7 containing 0·5 μg. adrenaline per c.c. (1/2,000,000). About 3 hr. later, the adrenaline is completely oxidized to adrenochrome.

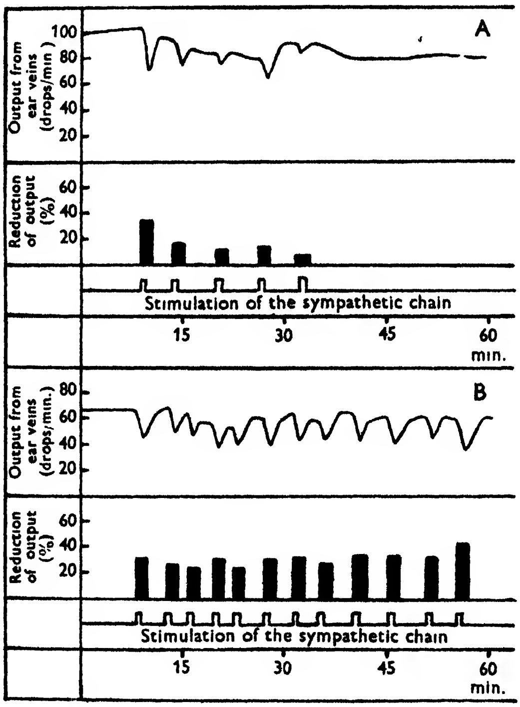


Fig. 1. A, exhaustion of the chemical promediator stock by repeated stimulation of the cervical sympathetic chain, the perfusing solution containing neither adrenochrome nor any substance able to supply it. B, failure to produce exhaustion when perfusing with a solution containing adrenochrome (0·5 μg./c.c.).

RESULTS

The chemical promediator of the sympathetic nervous system

Exhaustion of chemical promediator stock by repeated stimulation. Pissemski's perfusion of the ear enabled us to show the disappearance of the response to the sympathetic after repeated stimulation (Fig. 1 A). At the start of the

experiment, stimulation of the cervical sympathetic for 45–60 sec. caused a striking decrease in the number of drops. The vaso-motor response became progressively less, until it finally disappeared after 5–10 periods of stimulation and sometimes even earlier.

We have assumed that each such series of repeated stimuli transforms some of the chemical promediator into the mediator proper. As the stimuli were repeated, the stock of chemical promediator was used up and less mediator was discharged.

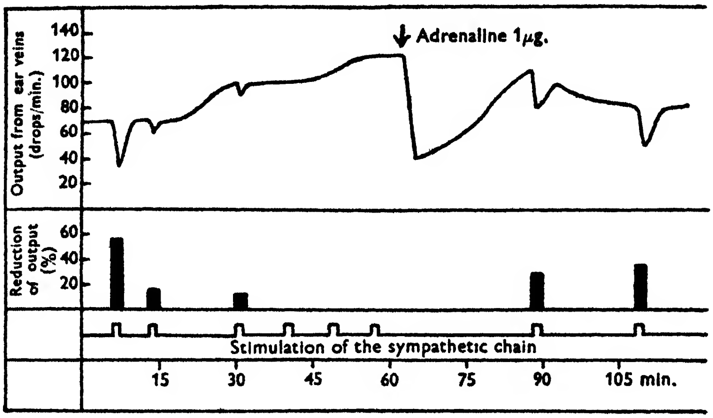


Fig. 2. Restoration of sympathetic excitability by adding adrenaline (1 μg.) to the perfusion fluid.

Restoration of sympathetic excitability by adding adrenaline to the perfusion fluid. After complete disappearance of the response we injected 1 μg. adrenaline into the perfusion fluid. After a strong vaso-constriction lasting about 10 min., stimulation of the sympathetic chain again caused a decreased flow in the perfused ear (Fig. 2).

We have assumed that the renewed response to the sympathetic is due to the transformation of the injected adrenaline into adrenochrome (or some closely related oxidation product of adrenaline), and a consequent reconstitution of the stock of chemical promediator.

Inexhaustibility of the promediator by perfusion with a solution containing adrenochrome. Adrenochrome (0·5 μg./c.c.) was added to the perfusion fluid before we tried to exhaust the stock of chemical promediator by repeated stimulation of the sympathetic chain after perfusion for 30 min.

Under these conditions we could not obtain any decrease of the vaso-motor response (Fig. 1 B).

Increase of the vaso-motor response by addition of adrenochrome to the perfusion fluid. In some experiments vascular anastomosis prevented pure Locke solution from perfusing the ear: a small quantity of blood was mixed with the perfusion fluid.

In these, as in the preceding experiments, we could not obtain a disappearance of the vaso-motor response after repeated stimulation, probably because the circulating blood carries oxidized adrenaline to the extremities of the sympathetic system. Nevertheless, in one of our experiments, we noticed during the stimulation an average reduction of 29 % in the perfusion flow through the ear after 18 periods of stimulation in 45 min. After adding 0·5 μg./c.c. adrenochrome to the solution, the reduction increased to 43 %. Thus, adrenochrome increased by some 50 % the vaso-motor effect of the sympathetic stimulation (Fig. 3).

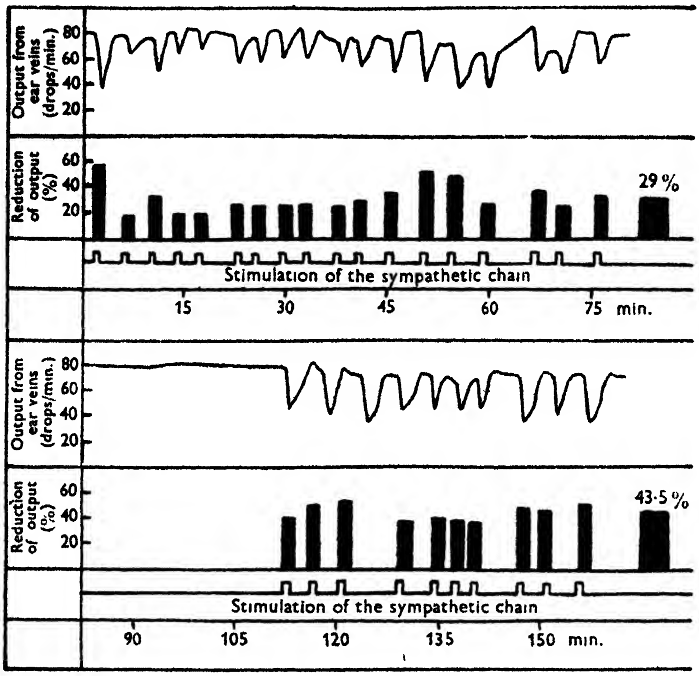


Fig. 3. Perfusion contaminated with blood. Increase of the vaso-motor sympathetic response by addition of adrenochrome (0·5 μg.) to the perfusion fluid.

Negative results with monoxime and monosemicarbazone of adrenochrome. When the response to sympathetic stimulation had completely disappeared, we tried to restore it by reconstituting the chemical promediator stock.

For this purpose we used two stable derivatives of adrenochrome, the monoxime (Green & Richter, 1937; Veer, 1942) and the monosemicarbazone (Braconier, Le Bihan & Beaudet, 1943) of adrenochrome. These substances are without any sympathomimetic effect (Bacq & Derouaux, unpublished). Both reduce the bleeding time in rabbits after rather a long period (Derouaux, 1943).

Addition of 0·5 μg. monoxime or monosemicarbazone of adrenochrome per c.c. to the perfusion fluid did not restore the response to the sympathetic.

Adrenochrome is the chemical promediator of the post-ganglionic fibres. As was to be expected, it is impossible to restore the normal effect of sympathetic stimulation by addition of monoxime or monosemicarbazone of adrenochrome, because of the stability of these substances in vitro and the latent period of their haemostatic action.

Since adrenochrome reduces the bleeding time without any delay, we were induced to suppose that this substance, or a very closely related component, is acting as a chemical promediator.

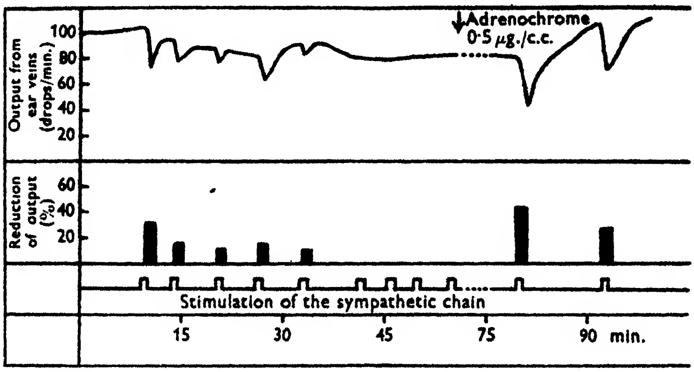


Fig. 4. Restoration of effect of sympathetic stimulation by adding adrenochrome to the perfusion fluid.

In order to prove this, we exhausted the response of a perfused ear to the sympathetic by repeated stimulation. Then we tried to revive it by addition of adrenochrome to the perfusing solution in a concentration of 0·5 μg./c.c. This did not alter the perfusion flow, but restored the response to the sympathetic, exhausted by repeated stimulation. Before perfusing the ear with adrenochrome, stimulation of the cervical sympathetic chain did not reduce the venous outflow; afterwards the same stimulation reduced it greatly (Fig. 4).

DISCUSSION

The site of origin of the chemical adrenergic mediator and the conditions of its discharge

The rabbit's ear perfused with Locke solution progressively loses its response to sympathetic stimulation. It can be restored by adding adrenochrome in a concentration of 0·5 μg./c.c. to the perfusing fluid. In the same concentration, monoxime and monosemicarbazone of adrenochrome are inactive. These facts lead to the conclusion that the discharge of the chemical adrenergic mediator by stimulation of the post-ganglionic fibres cannot take place unless there is a promediator present at their peripheral ends. The complex constitutional formula of adrenaline also suggests the existence of such a promediator, for

there is no glandular structure that is capable of synthesizing the β (3-4-dioxyphenyl)-ethanol-methylamine from simpler elements at the ends of the sympathetic nerves.

Our experiments clearly indicate the presence of such a chemical promediator akin to adrenaline (cf. Bacq, 1935) and strongly suggest that the promediator is adrenochrome, or a closely related compound.

The experiments also have a bearing on another problem, namely, whether sympathin is discharged by the ends of the post-ganglionic fibres or by the cells which they supply.

Either thesis has supporters of note: the first, Parker (1932), Bacq (1935) and Dale (1935); the second, Cannon (1933), though his arguments are less strong. Supposing the ends of the nervous fibres enter the smooth muscle cells, Cannon thinks that 'a secretion from these minute twigs could not directly enter the blood and thus influence distant organs, but would first mingle with the fluid of the cell. In that case the escaping humoral agent would certainly be in part of muscular origin.' Comparative physiology (Parker, 1932) and embryology (Bacq, 1935) would be in favour of the nervous origin of the chemical adrenergic mediator for the ganglionic sympathetic cells and the chromaffin cells of mammals have the same embryological origin. The same fundamental function may therefore be attributed to both: namely, the discharge of adrenaline when stimulated by the pre-ganglionic fibre. Finally, Dale judiciously remarks that 'the permanent association of a particular neurone with one kind of transmission would be more easily interpreted if the transmitting mechanism were a part of the nerve ending'.

The arguments for the sympathetic origin of the adrenergic chemical mediator are certainly attractive, but they are not supported by the data we have collected. We have shown that the sympathetic excitability of a definite organ, namely the ear, depends on the presence in this organ of a stock of adrenochrome, acting as an adrenergic chemical promediator. Furthermore, the fact has previously been established that an injection of adrenochrome shortens the bleeding time for several hours (Derouaux, 1941 *a*, *b*). It is, on the contrary, unable to modify the arrest of an active haemorrhage (Roskam & Derouaux, 1944).

Several of our previous findings (Derouaux & Roskam, 1937; Roskam, 1938; Derouaux, 1941 *a*-*d*, 1942 *a*, *b*) suggest that spontaneous haemostasis is under the direct control of the local sympathetic nervous system, as is also the reaction of blood vessels to pricking or to cutting (Magnus, 1923, 1924; Herzog, 1925; von Bernuth, 1925; Heimberger, 1925; Leschke & Wittkower, 1926; Macfarlane, 1941; Chen & Tsai, 1947; Hugues, 1947) which are certainly factors affecting bleeding time.

These facts agree with our hypothesis: the mechanical stimulation produced by cutting skin, perivascular tissues and blood vessels, has a local vaso-

constrictor effect due to a local discharge of adrenergic chemical mediator or sympathin. At the peripheral extremities of the sympathetic nervous system, there is a stock of adrenergic chemical promediator. The larger this supply the greater the discharge of sympathin. Previous injection of adrenaline, of sympathomimetic amines or of adrenochrome increases the supply of promediator and hence the vasoconstrictor response to any stimulus.

Derouaux (1941*b*) observed, however, that in a rabbit whose ears were denervated 4 weeks previously, an injection of 1 μg. adrenaline retains its haemostatic action, and this lasts, as in a normal animal, for 15, 30 and 60 min. As previously stated, this action is certainly due to an oxidation product of adrenaline, adrenochrome or a closely related derivative. Since, 4 weeks after the extirpation of the superior cervical ganglion, the post-ganglionic fibres have undoubtedly degenerated, it must be concluded that the discharge of sympathin can occur without any participation of the sympathetic nervous system.

There appear to be two possible explanations: either a tissue between the nerve fibre and the peripheral cell and not degenerating after nerve section is responsible for the phenomena we have studied; or the peripheral cell, supplied with a post-ganglionic fibre, is able to store the chemical promediator and then to turn it into sympathin without the help of the sympathetic system. We consider the second hypothesis the more likely and of some importance from a theoretical point of view. We believe that the conversion of the inactive chemical promediator into active sympathin takes place in the cells supplied with post-ganglionic fibres, usually following a stimulation of the latter. It could, however, be the result of a direct stimulation of the peripheral cell itself.

SUMMARY

1. Observations on spontaneous haemostasis raised the question whether adrenochrome or a closely related compound is the promediator of the chemical transmission of the adrenergic nervous impulses. In order to solve that problem, ears of rabbits were perfused with Locke solution without interrupting their nervous supply.

2. Under these conditions the vascular effect of sympathetic stimulation was progressively abolished, but was restored by the addition of adrenaline or adrenochrome to the perfusion fluid.

3. The response was maintained if the perfusion fluid contained adrenochrome.

4. Stable derivatives of adrenochrome did not restore the effect of sympathetic stimulation.

5. These experiments suggest that the adrenergic chemical promediator is adrenochrome or a closely related compound.

6. Since spontaneous haemostasis is governed by the sympathetic system,

and the haemostatic action of adrenaline, due to adrenochrome, persists in a rabbit whose ears were denervated 4 weeks before the experiment, it must be concluded that the storage of adrenochrome (or of the closely related compound acting as adrenergic promediator) and its conversion into sympathin may occur in the cells supplied by post-ganglionic fibres.

We are indebted to the 'Patrimoine de l'Université de Liége' for a grant in aid of this investigation and to Major F. J. Lewy, U.S. Army Medical Corps, who assisted in the translation of the paper.

REFERENCES

Armstrong, C. W. J., Cleghorn, R. A., Fowler, J. L. A. & McVicar, G. A. (1939). *J. Physiol.* **96**, 146.

Bacq, Z. (1933). *Arch. int. Physiol.* **36**, 167.

Bacq, Z. (1935). *Ergebn. Physiol.* **37**, 82.

Bacq, Z. & Derouaux, G. *Arch. int. Pharmacodyn.* (in preparation).

Braconier, Fr., Le Bihan, H. & Beaudet, C. (1943). *Arch. int. Pharmacodyn.* **69**, 181.

Burn, J. H. (1932). *J. Physiol.* **75**, 144.

Cannon, W. B. (1933). *Science*, **78**, 43.

Chen, T. I. & Tsai, C. (1947). *XVII Int. Physiol. Congr. Oxford*, Abstr. Comm. p. 366.

Coombs, H. (1925). *Amer. J. Physiol.* **72**, 200 *P.*

Dale, H. H. (1935). *Proc. R. Soc. Med.* (*Sect. Therap. and Pharm.*), **28**, 15.

Derouaux, G. (1941 *a*). *Arch. int. Pharmacodyn.* **65**, 125.

Derouaux, G. (1941 *b*). *Arch. int. Pharmacodyn.* **66**, 202.

Derouaux, G. (1941 *c*). *Arch. int. Physiol.* **51**, 269.

Derouaux, G. (1941 *d*). *Arch. int. Pharmacodyn.* **66**, 231.

Derouaux, G. (1942 *a*). *Verh. K. Vlaamsche Akad. Geneesk. V. Belgie*, **4**, 95.

Derouaux, G. (1942 *b*). *Arch. int. Pharmacodyn.* **68**, 311.

Derouaux, G. (1943). *Arch. int. Pharmacodyn.* **69**, 142.

Derouaux, G. & Roskam, J. (1937). *J. Physiol.* **90**, 65 *P.*

Elliott, T. R. (1904). *J. Physiol.* **31**, 20 *P.*

Green, D. E. & Richter, D. (1937). *Biochem. J.* **21**, 546.

Heimberger, H. (1925). *Klin. Wschr.* **27**, 1301.

Herzog, F. (1925). *Pflüg. Arch. ges. Physiol.* **207**, 476.

Hugues, J. (1947). *C.R. Soc. Biol., Paris*, **141**, 1154.

Leschke, E. & Wittkower, E. (1926). *Z. klin. Med.* **111**, 649.

Macfarlane, R. G. (1941). *Quart. J. Med.* **10**, 1.

Magnus, G. (1923). *Arch. klin. Chir.* **125**, 612.

Magnus, G. (1924). *Arch. klin. Chir.* **130**, 237.

Parker, G. H. (1932). *Humoral Agents in Nervous Activity with Special Reference to Chromatophores.* London: Cambridge University Press.

Pissemski, A. (1914). *Pflüg. Arch. ges. Physiol.* **156**, 426.

Roskam, J. (1938). *Arch. int. Physiol.* **47**, 325.

Roskam, J. & Derouaux, G. (1944). *Arch. int. Pharmacodyn.* **69**, 348.

Secker, J. (1938). *J. Physiol.* **94**, 259.

Veer, W. L. C. (1942). *Rec. Trav. chim. Pays-Bas*, **61**, 638.

von Bernuth, F. (1925). *Klin. Wschr.* **17**, 830.

J. Physiol. (1949) 108, 9–11 612.127.2

NEW METHOD FOR MEASURING THE ARTERIO-VENOUS OXYGEN DIFFERENCE BY MEANS OF PHOTOELECTRICAL COLORIMETER

By B. ISSEKUTZ, Jr., G. HETÉNYI, Jr. and I. FEUER

From the Physiological Department of the University of Szeged, Hungary

(*Received* 15 *October* 1947)

The methods elaborated for measuring the oxygen content of the blood are founded either on reduction of the oxyhaemoglobin or on the oxidation of the reduced haemoglobin by shaking it with air. From the change of the light extinction it is possible to estimate the oxygen content, in the first case directly, in the second after establishing the total haemoglobin content of the blood sample (Kramer, 1935; Issekutz, Jr., 1941). Usually, as in measurements of the metabolic rate or the cardiac output, the difference of the oxygen concentration of the arterial and venous blood is required rather than the absolute oxygen content of the blood. Therefore it seemed desirable to elaborate a method for direct measurement of the arterio-venous oxygen difference. The first condition of such a method is that the difference of the extinctions of the arterial and venous bloods should depend solely on the concentration of the reduced haemoglobin, that is, it should be independent of the absolute haemoglobin content of the blood. This condition is necessary in order to elaborate an extinction curve of general validity, and this can only be done if Beer's law is valid for oxyhaemoglobin as well as reduced haemoglobin. This is the case in the red part of the spectrum (between 6000 and 6500Å.), where the absorption coefficients of both the reduced haemoglobin and the oxyhaemoglobin are nearly constant. By measuring in this band, with filter RG_1 (Schott u. Gen. Jena), the advantage of monochromatic light is obtained.

According to Beer's law the extinction of the arterial blood is

$$E_1 = \epsilon_o c_{o1} L + \epsilon_r c_{r1} L, \tag{1}$$

where ϵ_o and ϵ_r are the extinction constants of the oxyhaemoglobin and of the reduced haemoglobin, c_{o1} and c_{r1} are the concentrations of the oxyhaemoglobin and reduced haemoglobin in the arterial blood, and L is the length of the path of light through the absorption cell.

The extinction of the venous blood is

$$E_2 = \epsilon_o c_{o2} L + \epsilon_r c_{r2} L, \tag{2}$$

where c_{o2} and c_{r2} are the concentrations of the oxyhaemoglobin and the reduced haemoglobin in the venous blood. The arterio-venous oxygen difference is

$$E_2 - E_1 = L\,(\epsilon_o c_{o2} + \epsilon_r c_{r2} - \epsilon_o c_{o1} - \epsilon_r c_{r1}). \tag{3}$$

Assuming that the total haemoglobin concentrations of the arterial and venous bloods are equal, i.e. that

$$c_{o1} + c_{r1} = c_{o2} + c_{r2}, \tag{4}$$

(3) becomes

$$E_2 - E_1 = L\,(\epsilon_r - \epsilon_o).(c_{r2} - c_{r1}). \tag{5}$$

As the length of path and the difference of the extinction coefficients are constants, the extinction difference of the arterial and venous bloods depends only on the difference of their reduced haemoglobin contents.

METHOD

The correctness of this calculation was demonstrated as follows: from the vena cubitalis of different persons approximately 10 ml. of blood were collected under paraffin oil. To prevent coagulation and to haemolyse the blood, 1 ml. 3% saponin (saponinum puriss, Merck) and 2% Calgon solution were added. After total haemolysis, part of the blood was saturated with oxygen, by shaking it with air, and transferred to the absorption cell ($L=1$ mm.) of Havemann's photocolorimeter (Havemann, 1939, 1940, 1941). A similar absorption cell was filled with blood not shaken with air. The first cell was put into the colorimeter, the measuring drum of which was set to 100, and with the compensating photoelement the galvanometer was brought to zero. The first cell was then replaced by the second, and the distance through which it was necessary to turn the drum so that the galvanometer returned to zero again was measured. The extinction corresponding to this value was obtained from a table attached to the colorimeter. By subtracting the value corresponding to 100 we obtain the value of $E_2 - E_1$.

To determine the amount of reduced haemoglobin to which this value corresponds, it is necessary to determine how much oxygen the venous blood is able to take up. This was estimated in Warburg manometers by the Haldane-Barcroft method, according to Gibson's modification (Gibson, 1943).

RESULTS

By this method we determined the extinction difference due to the oxygenation and the oxygen-absorbing ability of several blood samples of different haemoglobin concentrations and different venosity from different persons. The absorbed oxygen (cu.mm./1 ml. blood) was plotted against the extinction differences in Fig. 1. The result was a well-defined extinction curve, though each point was obtained from the bloods of different persons. This proves that the extinction difference is independent of the absolute haemoglobin concentration and depends only on the oxygen uptake or, in other words, on the concentration of the reduced haemoglobin, and on the change of the value of $\epsilon_r - \epsilon_o$. This value increases as may be seen from the curve relating the extinction to the increasing quantity of reduced haemoglobin.

As controls, nine experiments were performed on anaesthetized dogs. Blood samples were taken both from the femoral artery and vein, their oxygen uptakes were determined by the gasometric method, and the oxygen differences

thus obtained directly. At the same time, from the same blood samples, the arterio-venous oxygen difference was determined by the colorimetric method. The results are summarized in Table 1.

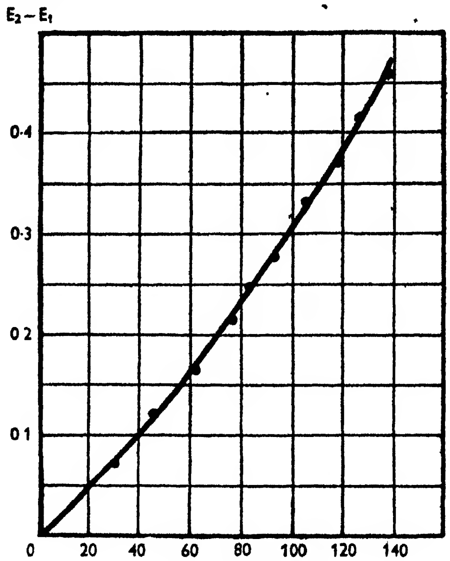


Fig. 1.

TABLE 1

Case	Oxygen uptake of blood (cu.mm./ml.)		Arterio-venous O_2 difference (cu.mm./ml. blood)		Deviation (%)
	Arterial	Venous	Gasometric	Colorimetric	
1	5·5	127·5	122	119·5	−2·5
2	7·5	58·5	51	54	+5·9
3	8·5	98	89·5	92	+2·8
4	2	82·5	80·5	79·5	−1·2
5	8·5	143	134·5	131	−2·6
6	1	65·5	64·5	63	−2·3
7	1	58·5	57·5	56	−2·6
8	0	91·5	91·5	88·5	−3·3
9	16	125	109	111	+1·8

By means of the extinction curve the extinction difference of the arterial and venous blood, haemolysed as described above, gives directly the arterio-venous oxygen difference. By this method a difference of 0·5 cu.mm. O_2/ml. blood can easily be measured.

REFERENCES

Gibson, J. (1943). *J. Physiol.* **102**, 83.

Havemann, R. (1939). *Biochem. Z.* **301**, 105.

Havemann, R. (1940). *Biochem. Z.* **306**, 224.

Havemann, R. (1941). *Angew. Chem.* **54**, 105.

Issekutz, Jr., B. (1941). *Arch. exp. Path. Pharmak.* **197**, 332.

Kramer, K. (1935). *Handbuch der Arbeitsmethoden*, Abt. V, p. 1098.

J. Physiol. (1949) 108, 12–23 612.111.22

A SIMPLIFIED METHOD FOR THE DETERMINATION OF CIRCULATING RED-CELL VOLUME WITH RADIOACTIVE PHOSPHORUS

By E. B. REEVE and N. VEALL*

From the Clinical Research Unit, Guy's Hospital and the Radiotherapeutic Research Unit, Hammersmith Hospital

(*Received* 6 *December* 1947)

INTRODUCTION

Hevesy and his colleagues developed a method for labelling red blood cells in vitro with the radioactive phosphorus isotope P_{32}, and they applied this method to the estimation of the total volume of the circulating red cells (Hevesy & Zerahn, 1942; Hevesy, Köster, Sorensen, Warburg & Zerahn, 1944). It has also been used to estimate the rate of mixing in the circulation under various conditions (Nylin, 1945). A few ml. of the experimental subject's blood are incubated for 2 hr. with a few μC. of P_{32}, and about half of the P_{32} passes into the red cells. A measured volume of this radioactive whole blood is injected into the subject and samples of blood are drawn at intervals. The uninjected remainder of the radioactive blood and the blood samples are spun at high speed (5000 r.p.m.), the supernatant plasma is sucked off, and the remaining packed red cells, which contain about 3% of their total weight of trapped plasma, are weighed and wet-ashed. The phosphorus is precipitated from solution as magnesium ammonium phosphate, and the radioactivity of the precipitate is estimated with a Geiger-Müller counter. The advantages of the method are that P_{32} is a radioactive element relatively easy to procure; that accurate radioactivity measurements can be carried out with little difficulty; and that small volumes of the subject's own red cells can be marked in vitro and then reinjected.

It seemed that this method might be considerably simplified if it were possible (*a*) to inject radioactive red cells without radioactive plasma and (*b*) to avoid the wet-ashing of packed red cells and precipitation of phosphorus. The objections to the injection of plasma containing P_{32} are that the subject's whole-blood samples, since they contain P_{32} in the plasma, cannot be used for

* Working on behalf of the Medical Research Council.

analysis; that in the body some of the plasma P_{32} runs into the red cells, and after the withdrawal of blood samples there is a possibility of further exchanges of P_{32} between red cells and plasma; and lastly, that double the required dose of radiation is administered to the subject. Although the radiation hazards in these experiments appear to be negligible, as will be shown later, it is felt that until more experimental data are available, the quantity of P_{32} used should be kept as low as possible, particularly if several measurements are needed on the same subject. The objections to the wet-ashing and precipitation are that any errors in these processes are added to the other errors of the method.

Experiments have therefore been carried out to establish and to test a method in which the radioactive red cells were washed free from radioactive plasma before they were injected, and in which the radioactivity measurements were carried out on liquid blood samples by means of a specially designed counter.

METHOD

General. 5–10 ml. of the experimental subject's blood are drawn, heparinized, and incubated for 1 hr. with, for example, 7 μC. of P_{32} in a specially designed centrifuge tube. The resulting radioactive red cells, which contain about 2 μC. of P_{32}, are washed free from their radioactive plasma by three washings with ice-cold physiological saline. A suspension of these cells in saline is injected into the subject and venous samples are drawn at intervals after the injection. The whole-blood samples, and a small portion of the suspension of the radioactive red cells injected, which is to be used as a standard, are diluted with a phosphate-citrate mixture and the red cells are lysed with saponine. The dilution of the standard is sufficient to reduce its radioactivity to about the same level as that in the samples. The radioactivity of the resulting solutions is recorded in turn by a specially designed Geiger-Müller counter. The haematocrits of all blood samples and the suspension of radioactive red cells are estimated by spinning them in 10 cm. haematocrit tubes for 30 min. at 3000 r.p.m. in a centrifuge of 15 cm. radius (applied force $=1500\times g$).

The R.B.C. volume in ml. is derived from the following equation:

$$\text{R.B.C.V.} = AH/n,$$

where A = the total quantity of radioactivity in the *red cells* injected into the subject, n = the quantity of radioactivity in 1 ml. of the subject's whole blood, after the injection of the radioactive red-cell suspension, corrected for loss of P_{32} from the blood, and H = the volume occupied by the red cells in 1 ml. of the subject's blood. The methods of estimating the values of A and n are described later. H is determined from the formula $H = Ht \times 0{\cdot}95$, where Ht is the haematocrit value determined as described above, and 0·95 is the correction applied for the plasma trapped in the packed cell column (Barnes, Loutit & Reeve, 1948).

Washing the red cells. The following method of washing is satisfactory. It should be emphasized that all manipulations of blood or red cells must be done very gently.

Before the experiment 0·05 ml. of strong heparin solution (B.D.H., 5000 i.u./ml.) and 0·1–0·3 ml. of a solution containing 3–10 μC. of P_{32} as Na_2HPO_4, made isotonic with added NaCl, are placed in a special glass-stoppered centrifuge tube of volume about 30 ml. (Fig. 1), which is then autoclaved at a pressure of 15 lb./sq.in. for 30 min. Such tubes may be prepared several days before use, as long as allowance is made for radioactive decay when the phosphorus is added.

8–10 ml. of the subject's blood are drawn into a dry sterile syringe, a fresh needle is fitted to the syringe, and the blood is gently squirted into the centrifuge tube. The tube is then stoppered and the contents are mixed without wetting the stopper. The centrifuge tube is then placed in an incubator at 38° C., lying on its side, but tilted sufficiently to avoid wetting the stopper, and rotated gently at about 10 r.p.m. for 1–2 hr. on a pair of motor-driven rollers.

The rate of passage of P_{32} between red cells and plasma is very slow at temperatures near 0° C., so during the washing process the red cells are kept cold. While the incubation is proceeding, a bottle containing 200 ml. of sterile 0·9 % NaCl is cooled in iced water. When incubation is finished the centrifuge tube is also cooled. As soon as the blood is cold about 20 ml. of ice-cold saline are added

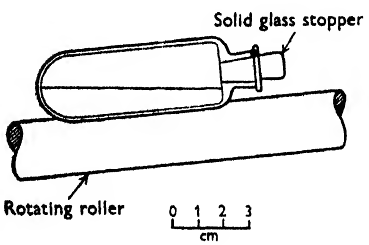


Fig. 1.

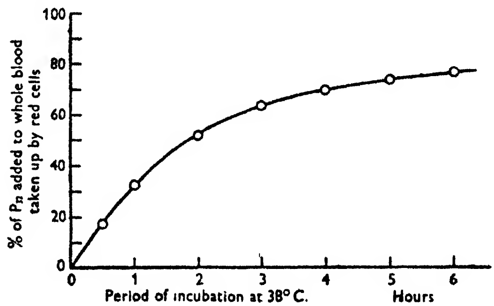


Fig. 2.

Fig. 1. The stoppered centrifuge tube, drawn to scale, in which the blood is incubated with P_{32}, and the red cells are washed free of plasma.

Fig. 2. The rate of uptake of P_{32} by the red cells when normal whole blood is incubated for varying times with P_{32}.

to it; the blood and saline are gently mixed and centrifuged for 5 min. at 2000 r.p.m. in a centrifuge with a radius of 14 cm. The supernatant mixture of saline and radioactive plasma is sucked off through a sterile Pasteur pipette (made narrow to give a slow flow), which is attached by sterile rubber tubing via a trap bottle to a suction pump. About 23 ml. of the cold sterile saline are now added, gently mixed with the red cells, and the red cells are again spun down as before. After a further 23 ml. of cold saline have been added, mixed and sucked off in this way, a final lot of about 23 ml. is added but not mixed, and the centrifuge tube is returned to the iced water. Mixing is delayed until the red cells are about to be drawn into the syringe for injection, and then care should be taken to displace and mix the red cells which adhere to the bottom of the centrifuge tube. It is desirable to limit the time between completion of the washing and the injection to 15–20 min.

Ideally the injected suspension of red cells should contain all its radioactivity in the red cells. Routine estimations of the P_{32} content of the red cells and supernatant saline show that the P_{32} content of the saline averages 1 % of that of the red cells. The highest saline activity found, on one occasion only, was equivalent to 2·2 % of the activity of the red cells.

Period of incubation and radioactivity in red cells. Red-cell suspensions of approximately known radioactivity can be prepared. Fig. 2 shows the percentage of P_{32} found in human red cells after varying periods of incubation, when a small amount of isotonic solution containing P_{32} is added to normal whole blood. It is typical of the results obtained in several experiments. About

30% of the added P_{32} passed into the red cells in the first hour, and about 50% in 2 hr. The curve shown may be used to predict the approximate percentage of added P_{32} that will enter the red cells in a given period of incubation at 38° C., when volumes of up to 0·5 ml. of isotonic solution containing P_{32} are added to 6–10 ml. of normal freshly drawn whole blood.

Hevesy *et al.* (1944), and the users of his method, have incubated blood with P_{32} for 2 hr., which is near the satisfactory minimum time for his method. For, in Hevesy's method, the shorter the period of incubation, the greater is the proportion of P_{32} injected in the plasma of the radioactivated blood, and hence the greater the errors caused by the presence of radioactive plasma. It is an advantage of the washing method that shorter periods of incubation may be used, thus reducing the time taken in preparation.

Rate of loss of P_{32} *from the red cells in vitro and in vivo.* It is necessary to show that the incubation and washing does not damage the red cells either so that they are lysed and removed, or so that they readily lose their contained P_{32} on injection into the circulation.

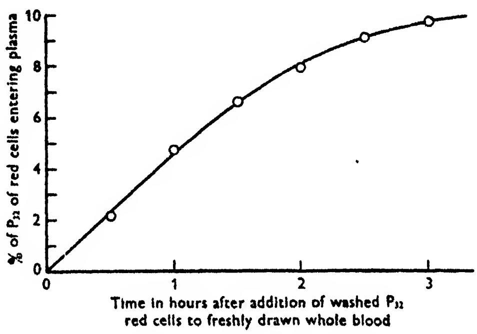


Fig. 3. The rate of loss of P_{32} from washed red cells containing P_{32}, prepared as described in the text, when they are added to five times their volume of whole blood.

Experiments have been made in vitro and in vivo to estimate the rate of loss of P_{32} from such washed red blood cells. 5 ml. of washed red cells containing P_{32} were rotated in an incubator at 38° C. with 25 ml. of the subject's freshly drawn inactive whole blood, samples of the mixture were withdrawn at intervals, and the P_{32} content of these and of the plasma separated from them estimated. Fig. 3 shows that there is a slow loss of P_{32} to the plasma.

P_{32} is also slowly removed when washed red cells are injected into the circulation. Measurements have been made of the P_{32} content of serial samples drawn after injections of radioactive red cells in man. In Fig. 4 are shown some of the results of a series of such estimates. The rate of loss of P_{32} in the first hour is slow and varies slightly in different subjects. Our estimates show values varying between 3 and 13% of the injected P_{32} for the loss in the first hour, with a mean loss rate of 6–7%. For blood-volume estimates this is not a serious rate of loss.

It would be a great advantage if a suspension of red blood cells could be prepared from which the loss of P_{32} is negligible during the first few hours after injection. The best suspensions used have shown a loss rate calculated to be 3% of the total per hour, which is barely within the error of the method of estimation. These were prepared by incubating the blood with P_{32} of high specific activity for 1 hr. and then washing as described. In a good part of this work the available phosphorus had only moderate or low specific activity. In Fig. 4 results obtained with P_{32} of moderate or low specific activity are plotted as continuous lines (in these experiments the red cells were incubated with the P_{32} for 2 hr.), and results obtained with P_{32} of high specific activity are plotted as dotted lines (in these experiments the red cells were usually incubated for 1 hr.). In seven experiments with P_{32} of low specific activity the average loss rate over the first hour from the time of injection was 9% of the total, and in five experiments with P_{32} of high specific activity the loss

was 4·5 % of the total. Some of the experiments quoted are shown in Fig. 4. These curves suggest that there might be a slower loss rate of P_{32} from cells prepared by the second procedure than from those prepared by the first, but more experimental data are necessary to establish this.

Measurement of radioactivity of samples. A Geiger-Müller counter which takes liquid samples was specially designed for these estimations. It has adequate sensitivity, a low background, is very simple in use, and requires about 10 ml. of solution to fill it. A full description is given elsewhere (Veall, 1948).

It has been found that plasma separated from blood samples drawn 10 min. or more after injections of the radioactive red-cell suspension, placed on ice (to slow down phosphorus exchange between red cells and plasma), and spun rapidly while cold, contain amounts less than 1–2 % of the total P_{32}. Hence all significant amounts of P_{32} are in the red cells. Therefore, in order to estimate the P_{32} it is only necessary to prepare an accurate dilution of the whole blood with a suitable

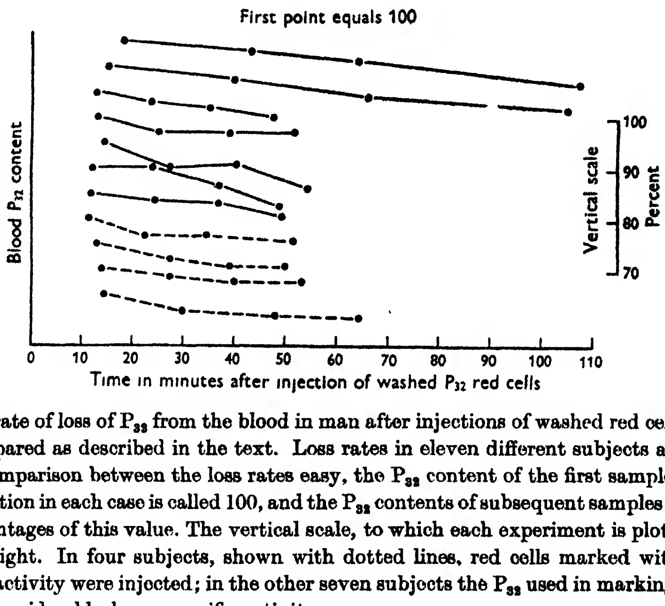


Fig. 4. The rate of loss of P_{32} from the blood in man after injections of washed red cells containing P_{32}, prepared as described in the text. Loss rates in eleven different subjects are shown. To make comparison between the loss rates easy, the P_{32} content of the first sample drawn after the injection in each case is called 100, and the P_{32} contents of subsequent samples are expressed as percentages of this value. The vertical scale, to which each experiment is plotted, is shown on the right. In four subjects, shown with dotted lines, red cells marked with P_{32} of high specific activity were injected; in the other seven subjects the P_{32} used in marking the red cells was of considerably lower specific activity.

diluting fluid, lyse the red cells with a small quantity of saponine, and pour the resulting solution into the counter, where its activity is measured. The haematocrit value of a portion of the same whole-blood sample is also determined. A diluting fluid which has been found to be suitable consists of 1 part of a 2·5 % sodium tricitrate solution and 9 parts of a M/15-Sorensen phosphate buffer solution of pH 7·3 (100 ml. of the latter is made from 76·8 ml. of M/15-Na_2HPO_4 and 23·2 ml. of M/15-KH_2PO_4).

Many other diluting fluids are satisfactory, but blood dissolved in a dilute ammonia solution, a diluent often used for blood, should be avoided. Blood so diluted, e.g. with 1·5 % ammonia, gels on keeping and becomes difficult to handle, and P_{32} appears to be lost slowly from solution, apparently being adsorbed on the glass walls of the containers.

With the phosphate-citrate solution no loss of P_{32} has been detected over 3–5 days when the natural decay rate is corrected for. 7 ml. of phosphate-citrate solution is usually added to 5 ml. of the subject's blood. At room temperature such samples soon become infected, so that if there is to be a delay of more than a few hours in measurement they are kept in a refrigerator. The red cells have not been lysed until just before the samples are to be measured.

Preparation of standards. Standards are prepared with the same order of radioactivity as the samples. In an average adult a satisfactory standard is made from 1 ml. of a 1/50 dilution of the radioactive red-cell suspension injected, 5 ml. of 'blank' blood and 6 ml. of phosphate-citrate mixture. This has the same order of radioactivity as a dilution of 5 ml. of the subject's 'active' blood with 7 ml. of phosphate-citrate solution. 1/50 dilutions of the radioactive red-cell suspensions are prepared from the uninjected remainder just before or after the injection, and are best made up in 50 ml. standard flasks. The 'blank' blood is drawn before the injection of the radioactive red cells. The use of 'blank' blood results in a standard which has the same composition as the blood samples with which it is compared, so that it is not necessary to determine and apply corrections to the observed counting rates for specific gravity variations.

Assuming that the very small amount of P_{32} contained in the saline of the suspension of red cells injected is rapidly removed from the circulation, it is desirable to correct the standard for its saline content of P_{32}. A part of the radioactive red-cell suspension which has been cooled in ice is spun rapidly while still cold, and a sample of the supernatant saline withdrawn for measurement of its P_{32} content. The haematocrit of the radioactive red-cell suspension is also determined. The P_{32} content of unit volume of the injected red cells (S_R) is then given by

$$S_R = S_W - S_S\left(\frac{100-H}{100}\right), \tag{1}$$

where S_W is the count obtained for the red-cell suspension, S_S is the count for the supernatant saline, and H is the per cent. red-cell volume of the injected suspension. (All counts are corrected for background, counter resolving time, and for the dilution of the sample.) As mentioned earlier the total P_{32} content of the supernatant saline, in our experience, averages 1 % of that of the red blood cells. Provided the method of washing and handling the radioactive red blood-cell suspension, is standardized the saline P_{32} content may be determined in a few experiments, and then, if sufficiently constant, assumed for later experiments. Where a high degree of precision is not being sought, this correction can be safely neglected.

In our experiments estimates have been made on duplicate or triplicate standards. Both 'blank' and 'active' blood samples have been heparinized. We have routinely corrected for the small amount of P_{32} contained in the saline of the red-cell suspension.

Calculation of red blood cell volume. The red blood cell volume (R.B.C.V.) is calculated from the equation

$$\text{R.B.C.V.} = S_R v/N, \tag{2}$$

where S_R = the P_{32} content of unit volume of the injected radioactive *red cells*, v = the volume of the injected radioactive red cells, and N = the P_{32} content of unit volume of the *red cells* in the subject's blood sample, corrected for loss of P_{32}, etc.

There is a gradual loss of P_{32} from the blood after an injection of radioactive cells. To determine the red blood cell volume therefore correction should be made for this loss. This may be done in each experiment by determining the P_{32} contents of a series of samples drawn at intervals after mixing of the injected red cells in the circulation is assumed complete, and extrapolating back to the time of injection through these concentrations on a graph in which concentration is plotted against time. Or, since the loss rates are neither great nor vary much, the P_{32} contents of one, but preferably two, samples, drawn 20–30 min. after the injection of the radioactive red cells (to allow adequate time for mixing), may be corrected for a mean loss rate determined in a series of subjects.

Thus in the R.B.C.V. estimates given in Table 1, a mean loss rate of 6 % of the total P_{32} per hour was taken, and samples drawn at 20 and 30 min. after injection were corrected accordingly. If n^{20} = the P_{32} content of 1 ml. of the 20 min. whole-blood sample and n^{30} = the P_{32} content of 1 ml. of the 30 min. whole-blood sample, then N^{20} and N^{30} of equation (2) are derived from the relationship

$$N^{20} = 1{\cdot}02 \times \frac{n^{20}}{H^{20}} \quad \text{and} \quad N^{30} = 1{\cdot}03 \times \frac{n^{30}}{H^{30}},$$

where $H = 0{\cdot}95 \times Ht$, and Ht is the observed haematocrit of 1 ml. of the sample.

RESULTS

Comparison of results with those given by the T 1824 haematocrit method. It is possible that in the first few minutes after the injection of radioactive red cells there is a rapid removal of P_{32}. This would not be detected by estimating the P_{32} content of serial samples of the blood drawn after mixing is complete. To test if it occurs, a comparison is required between estimates of red blood cell volume by the washed P_{32} red-cell method and an independent method.

Estimates of the red-cell volume obtained from the dye (T 1824) estimate of the plasma volume and the haematocrit are known to be too high, but recent work has shown that in normal subjects and some hospital patients these estimates tend to be too high by a fraction that does not vary greatly from subject to subject. Gibson, Peacock, Seligman & Sack (1946) have compared estimates of red-cell volume made with a carefully tested radioactive iron method of marking red cells with those obtained by T 1824 and the haematocrit in forty normal subjects. Neither their radioactive nor dye estimates were corrected for the plasma trapped in the spun haematocrit (see, for example, Gregersen & Shiro, 1938), and hence both are slight overestimates. If their published haematocrit values are multiplied by 0·95 and their radioactive and dye-haematocrit values are recalculated, then the ratio $\frac{\text{R.B.C. volume marked red cell}}{\text{R.B.C. volume dye haematocrit}}$ averages 0·87 (S.D. $\pm$ 0·056). Barnes, Loutit & Reeve (1948) have also made comparisons between estimates of red blood cell volume given by a method they have carefully tested, the improved Ashby method of Dacie & Mollison (1943), and T 1824 haematocrit estimates in twenty normals and selected hospital patients. In these experiments the average value found for the above ratio was 0·88 (S.D. $\pm$ 0·06), and for the eight normals alone 0·89. Hence two independent and probably reliable methods show close agreement in their measure of the mean size of the overestimate given by the T 1824 haematocrit method. It may be here noted that the carbon monoxide method of estimating the red blood cell volume gives estimates roughly the same size as the T 1824 haematocrit method (Root, Roughton & Gregersen, 1946). In view, however, of the possibility of escape of carbon monoxide from the circulation, which would cause an overestimate, or, if the carbon monoxide estimates are correct, the greater difficulty of explaining the consistently low results of Gibson *et al.* and Barnes *et al.*, the results of the latter workers are to be preferred. Further discussion will be found in Barnes, Loutit & Reeve (1948), and Reeve (1948).

If it is accepted that the estimates of red blood cell volume given by the radioactive iron and the Ashby methods are correct, in normal subjects, on the average, the dye-haematocrit estimate multiplied by a factor near 0·88 should give an estimate near the correct value. If the washed P_{32} red-cell method here described gives correct estimates of the red blood cell volume, then it

should also give estimates on the average about 0·88 of the dye-haematocrit estimates in a series of normal subjects.

In Table 1 are shown a series of comparisons between T1824 estimates of red blood cell volume and washed P_{32} red-cell estimates in normal, fasting, reclining subjects. Injections of dye and radioactive red cells were given within 3–5 min. of each other and then four samples of blood were drawn at 12–15 min. intervals and analysed for their plasma dye and P_{32} content. Dye estimates of the plasma volume were made as described in Barnes, Loutit & Reeve (1948), and the haematocrits were corrected by multiplying by 0·95. The values for red blood cell volume are calculated from the activities of the blood samples drawn at approximately 20 and 30 min. after the injection of the marked red cells. A correction is applied to each reading to allow for loss of P_{32} from the red blood cells, based on the mean value found for this series of 6% per hour.

TABLE 1. Comparison of red blood cell volume estimates by the Evans Blue T1824 haematocrit and washed P_{32} red-cell methods in normal fasting subjects

Subject	Height (in.)	Weight (lb.)	T1824 plasma volume (ml.)	Haematocrit corrected for trapped plasma	X Calculated R.B.C.V. T1824 and haematocrit (ml.)	Y Marked cell R.B.C.V. 20 min. sample (ml.)	Z Marked cell R.B.C.V. 30 min. sample (ml.)	Ratio Y/X	Ratio Z/X
A	67	142	3140	38·9	1995	1825	1750	0·92	0·88
B	72	154	3420	43·1	2590	2200	2200	0·85	0·85
C	66	133	2920	44·2	2310	2040	2040	0·89	0·89
D	73	166	3000	45·3	2480	2140	2110	0·86	0·85
E	70	148	3370	42·1	2450	2230	2210	0·91	0·90
F	72	164	3630	40·0	2420	2000	2050	0·83	0·85
G	66	147	3110	38·1	1910	1735	1680	0·91	0·88
H	69	148	3510	40·4	2380	2060	2120	0·87	0·89
I	71	165	3530	43·4	2710	2320	2290	0·86	0·85
J	70	150	3300	42·2	2410	2070	2140	0·86	0·89
K	73	166	3160	42·3	2320	2010	2000	0·87	0·86
L	73	162	3460	45·2	2840	2400	2400	0·85	0·85
M	76	207	4120	47·2	3690	3080	3050	0·83	0·83
							Mean	0·87	0·87

The mean ratio $\dfrac{\text{Washed } P_{32} \text{ red cell R.B.C.V.}}{\text{T1824 haematocrit R.B.C.V.}}$ is 0·87. Further, the scatter about the mean is not great, S.D. being $\pm 0{\cdot}025$. Hence it is probable that significant amounts of P_{32} do not escape in the first few minutes after injection of the radioactive red cells. If this is true it follows that these results provide further evidence of a mean value near 0·87 for the above ratio in normal subjects, and throw further doubt on the accuracy of the estimates obtained by carbon monoxide methods.

Accuracy. The accuracy of the method should be fairly high. The necessary manipulations in the method are simple, and if errors from in vivo causes are excluded, the accuracy will depend on the measurements of the volume of

radioactive red blood cells injected, the precision of dilution of standards and samples, and the number of counts made on the standards and samples. In our experience it is easier to inject precisely a volume of the order of 20 ml. rather than a volume of the order of 3–5 ml. In radioactive counting technique the standard deviation of the total count is given by the square root of the total number of counts recorded. Thus, if 10,000 counts are made on a sample, the standard deviation will be ± 100 or 1% of the total. If 1–2 μC. of P_{32} in red blood cells are injected into an average adult, 5 ml. samples of the subject's blood are drawn, diluted to 12 ml. with the phosphate-citrate mixture and poured into the counter described, counts of 10,000 are obtained in 30–60 min. The background count is of the order of 5% or less of the total count. Estimates have been made of the P_{32} content of duplicate dilutions of a series of blood samples, and they have been found to agree within the standard deviation calculated on the basis of the number of counts observed. Hence the errors involved in manipulating the samples and preparing dilutions add relatively little additional error to the statistical counting error.

As a further check, a series of five estimates have been made in triplicate of the volume of blood in standard Blood Transfusion Service pint transfusion bottles. 2–5 ml. of radioactive red-cell suspensions prepared from freshly drawn group homologous blood were added to a bottle of citrated human blood, which had been stored 6 weeks or longer and was unfit for transfusion; and after thorough mixing at room temperature, three samples of the mixture were taken and analysed for their radioactivity. In these experiments the technique for measuring blood volumes in man was duplicated as exactly as possible, and the total activity of the samples was in the range used in our in vivo experiments, except that in the fifth experiment the radioactivity was three times the usual. Table 2 shows the estimates given by the P_{32} method compared with the volumetrically measured volumes of blood. These results show good agreement. Such old stored blood provided a good test, for, since it contained a number of small fibrin clots which made pipetting difficult, and the red cells easily haemolysed, it was not easy to handle.

TABLE 2. Estimates by washed P_{32} red-cell method of known volumes of stored blood in transfusion bottles

Experiment	Measured blood volume (ml.)	Blood volume estimated with P_{32} red cells: Sample 1 (ml.)	Sample 2 (ml.)	Sample 3 (ml.)	% Difference of mean R.A. estimate from measured
1	526	528	518	525	−1
2	515	519	521	522	+1
3	517	528	533	533	+3
4	525	535	520	519	0
5	527	541	541	548	+3
				Mean % difference	1 ± 2

Radiation dosage to the tissues. Tissue radiation dosages are usually expressed in roentgen units, and one roentgen is defined as that quantity of radiation which produces one electrostatic unit of ions, of both signs, in 1 ml. of air at 0° C. and 760 mm. Hg. pressure. It is usually accepted that when tissues are exposed to continuous radiation, the radiation dosage should not exceed 0·1 unit a day.

The dose R, in equivalent roentgens, received by the tissues after an injection of P_{32} may be derived from the equation

$$R = \frac{3{\cdot}7 \times 10^4 e \rho_a \bar{V}_\beta T C}{0{\cdot}693 Wt} \quad \text{(Marinelli, 1942)},$$

where e is the charge on the electron ($4{\cdot}8 \times 10^{-10}$ e.s.u.), ρ_a is the weight of 1 ml. of air ($1{\cdot}293 \times 10^{-3}$ g.), $\bar{V}_\beta$ is the average energy of the β particles emitted by P_{32} ($6{\cdot}95 \times 10^5$ eV. (Marinelli, Brinckerhoff & Hine, 1947)), T is the radioactive half-life of P_{32} in seconds ($14{\cdot}3 \times 8{\cdot}64 \times 10^4$), C is the number of microcuries of P_{32} per g. tissue, when 1 μC. is defined as that amount of radioactive material which gives $3{\cdot}7 \times 10^4$ disintegrations per sec., and Wt is the energy required to produce one ion pair in tissue (32·5 eV., (Lea, 1946)).

From this equation it is seen that if 1 μC. of P_{32} is distributed in each gramme of tissue, the total radiation dosage delivered to the tissues by P_{32} during its complete radioactive life is 875 r. Since the radioactivity declines exponentially with time, the tissues will receive the highest rate of dosage during their first day of exposure. In a tissue that receives 1 μC. of P_{32} per g. the rate of dosage on the first day will be 41·5 r. per day, on the 15th day half this rate, and on the 80th day about 1 % of this rate.

In the method described very much smaller dosages than 1 μC. per g. tissue are given. Thus, assume that for an estimation of R.B.C.V. 2 μC. of P_{32} are injected into a subject with a total blood volume of, say, 5200 ml. If all the P_{32} remained in the blood for the first 24 hr. after injection, then the radiation dosage received by the blood during this period would be 0·016 r. Since, however, the P_{32} is removed from the blood, about half leaving in the first 24 hr., the dose received by the blood will be smaller than this. When the P_{32} leaves the blood part is taken up into the bones, part into the liver, part into other tissues, and part is excreted. When P_{32} is so redistributed the maximum concentration of it found in any one tissue is thought not to exceed six times its average concentration in the body. Neglecting losses by excretion, if 2 μC. of P_{32} are distributed evenly through the tissues of a 70 kg. subject, then the average radiation received in the first 24 hr. will be 0·0012 r., and the maximum radiation on redistribution six times this value, or 0·007 r. per day.

It seems, therefore, that even if several R.B.C.V. estimations are made on the same subject, there is little risk from the amounts of radiation given.

DISCUSSION

Most of the previous red-cell volume estimates with P_{32} in man have been made using the technique of Hevesy *et al.*, in which whole blood containing P_{32} in cells and plasma is injected. Nylin (1947), using this method, has also made observations on the persistence of P_{32} after such injections, and claims that there is very little loss of P_{32} for the first hour after an injection. His results are to some extent complicated by the presence of a considerable quantity of P_{32} in the injected plasma. Reference to Fig. 2 of Nylin & Pannier (1947) shows that an appreciable proportion of P_{32} remains in the plasma of the subject for at least 2 hr. after an injection of radioactivated whole blood, and from Hevesy's and our experiments one would expect about 25% of the plasma P_{32} to enter the corpuscles per hour. Hence Nylin's observations are a measure of the P_{32} content of the red cells which depends on (*a*) the P_{32} content of the injected red cells and the loss rate of P_{32} from these red cells and (*b*) the rate of entry of plasma P_{32} into the red cells.

As already mentioned it would be a great advantage if a suspension of red blood cells could be prepared which on injection showed a negligible loss of P_{32} over a few hours. The best suspensions used in these experiments which have been prepared by incubating blood with P_{32} of high specific activity for 1 hr. have shown a nearly negligible loss rate of P_{32}. It is possible that with another washing solution, or better manipulations, suspensions of red blood cells containing P_{32} might be prepared routinely which show a negligible loss of P_{32} for the first 1 or 2 hr. after injection, and perhaps for even longer. But the technique here described is adequate for precise estimates of total red-cell volume, and has the merit of simplicity. Anderson (1942) has also used washed red cells containing P_{32} for red blood cell volume estimations in rabbits, but gives few details of his technique.

SUMMARY

1. The total red-cell volume in man is measured from the dilution of injected washed red cells, labelled in vitro with P_{32}. The red cells are prepared by incubating whole blood with P_{32}, and afterwards washed almost free of their radioactive plasma.

2. Following injections of suspensions of such washed red cells, P_{32} is lost very slowly from the circulation.

3. The results given by the method agree with results given by other reliable methods. The method has also been checked in vitro.

4. A specially designed Geiger-Müller counter taking liquid samples much simplifies the necessary manipulations of standards and samples.

5. Apart from knowledge of radioactive technique, the method requires no great technical skill in its use.

We would like to thank Miss D. Leeson and Miss H. Pels for excellent technical assistance in this work.

REFERENCES

Anderson, R. S. (1942). *Amer. J. Physiol.* **137**, 539.

Barnes, D. W. H., Loutit, J. F. & Reeve, E. B. (1948). *Clin. Sci.* **7**, 135.

Dacie, J. V. & Mollison, P. L. (1943). *Lancet*, **1**, 550.

Gibson, J. G. 2nd, Peacock, W. C., Seligman, A. M. & Sack, T. (1946). *J. clin. Invest.* **25**, 838.

Gregersen, M. I. & Shiro, H. (1938). *Amer. J. Physiol.* **121**, 284.

Hevesy, G., Köster, K. H., Sorensen, G., Warburg, E. & Zerahn, K. (1944). *Acta med. Scand.* **116**, 561.

Hevesy, G. & Zerahn, K. (1942). *Acta physiol. Scand.* **4**, 376.

Lea, D. E. (1946). *Actions of Radiations on Living Cells*, p. 22. Cambridge University Press.

Marinelli, L. D. (1942). *Amer. J. Roentgenol.* **47**, 210.

Marinelli, L. D., Brinckerhoff, R. F. & Hine, G. J. (1947). *Rev. Mod. Phys.* **19**, 25.

Nylin, G. (1945). *Amer. Heart J.* **30**, 1.

Nylin, G. (1947). *Amer. J. Physiol.* **149**, 180.

Nylin, G. & Pannier, R. (1947). *Arch. Int. Pharmacodyn.* **73**, 401.

Reeve, E. B. (1948). *Nutrit. Abstr. and Rev.* **17**, 811.

Root, W. S., Roughton, F. J. W. & Gregersen, M. I. (1946). *Amer. J. Physiol.* **146**, 739.

Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.

J. Physiol. (1949) 24–32 615.761.21:612.217.1

THE EFFECTS OF GLUCOSE ON THE ACTION OF THE RAT DIAPHRAGM

By R. J. S. McDOWALL, W. MIECHOWSKI and A. Z. SHAFEI
From the Department of Physiology, King's College, London

(*Received* 16 *December* 1947)

The rat phrenic-diaphragm preparation described by Bülbring (1946) has been shown to give remarkably regular responses for many hours when stimulated with single shocks every 10 sec., but to ensure this, in most preparations, an adequate supply of glucose is essential as it is for the isolated heart and the isolated intestine. Bülbring used 0·2 % glucose, but because this concentration of glucose is known to depress the synthesis or release of acetylcholine (Feldberg, 1944–5) a further study appeared desirable, especially as preliminary experiments indicated that some preparations contracted better in glucose-free Tyrode solution (McDowall & Shafei, 1947).

METHODS

The general method used in Bülbring's experiments was adopted with two modifications. (1) The portion of the phrenic nerve applied to the electrodes was raised above the level of the fluid in the bath and kept moist with cotton-wool soaked in Tyrode solution. It was, therefore, not affected by changes in the fluid in the bath. (2) The tendon was attached to the lever by means of wire so that the muscle could be stimulated through its tendon, another electrode being at the base of the preparation. This enabled the preparation to be stimulated directly although, admittedly, such stimulation must include stimulation of the nerve elements within the muscle unless neuromuscular transmission is paralysed. A bath of 50 ml. was used because it was found that for some unexplained reason the preparation tended to deteriorate rapidly in smaller baths in spite of full oxygenation. Stimulation was effected by means of a neon-lamp stimulator as used by Bülbring, and by means of an electronic apparatus delivering square-wave stimuli of known voltage, frequency and duration.

The experiments consisted generally of replacing a Tyrode solution containing 0·2 % glucose with one containing no glucose at all or vice versa.

RESULTS

The effect of removal of glucose in the bath fluid varies in different preparations and under different conditions of the experiment. It may have no effect at all on the muscle response to nerve stimulation, it may cause depression or augmentation or a combination of both.

In a number of experiments, especially when old rats in good condition were used, removal of the glucose from the bath fluid had no effect on the nerve-

muscle response. In one experiment the diaphragm continued to contract in the glucose-free Tyrode solution for over 7 hr. without showing any sign of depression of the nerve-muscle response. No doubt the energy was supplied from the glycogen stores of the preparation, for if steps were taken to reduce them (see later) the nerve-muscle response became either depressed or augmented.

An immediate depression of the nerve-muscle responses as a result of removing the glucose from the Tyrode solution is very uncommon. The appearance of depression depends largely on the strength and frequency of nerve stimulation and the duration of the experiment. It is best seen with maximal stimuli and at a rate of over 6 per min. Under these conditions removal of the glucose may bring about depression of the nerve-muscle responses in 45 minutes, but usually it takes several hours to do so, as in the

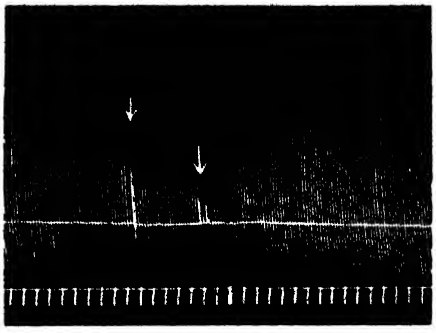


Fig. 1. A preparation which showed depression after being deprived of additional glucose for 2 hr. At the first arrow a control wash with Tyrode solution without glucose was made. At the second arrow glucose was added.

experiment of Fig. 1, in which the nerve-muscle responses became depressed 2 hr. after the glucose had been removed from the bath. On the readdition of the glucose to the Tyrode solution the nerve-muscle responses were at once restored (see Fig. 1). When with the same preparation the experiment was repeated, but using now a slower rate of nerve stimulation or submaximal stimuli, removal of the glucose was followed by pronounced augmentation of the nerve-muscle responses.

Augmentation of the nerve-muscle responses on removal of the glucose from the bath fluid could be obtained in varying degrees in about 70% of the preparations and in an even higher percentage when the diaphragms were from rats which had been used previously for dietetic experiments. The augmentation of the nerve-muscle responses is always transient, lasting for 5–20 min., and is followed by depression. Optimal stimuli for producing the effect are submaximal ones of long duration and at a rate of less than 6 per min. The best

effects were seen with submaximal stimuli using the neon stimulator or with square-wave stimuli of a duration of over 1400 μsec. Stimuli of such a duration are known to produce a response of the muscle which is repetitive although it is mechanically fused. Augmentation has, however, also been observed with stimuli of under 500 μsec. which do not produce a repetitive response (Brown & Burns, 1947). The augmentation is best seen in preparations which have been in the bath for 3–4 hr. and are thoroughly deprived of glucose or glycogen. The glucose deprivation may be assumed from the depression which follows the augmentation and which is so easily recovered from when glucose is added.

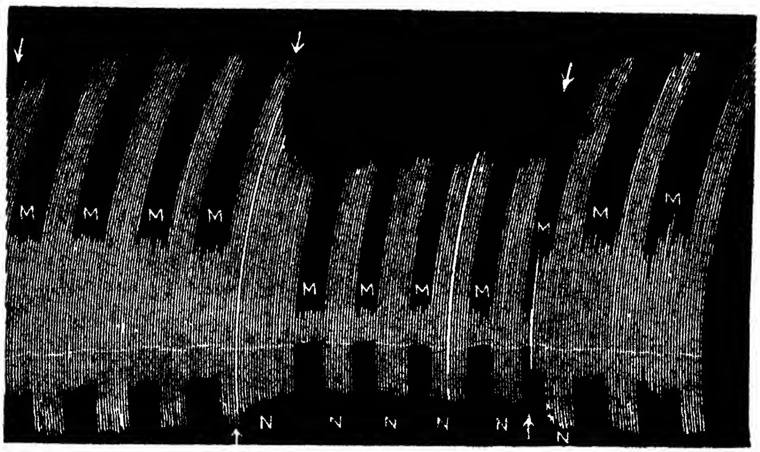


Fig. 2. Record of contraction of the diaphragm caused to contract by stimulation of the phrenic nerve (*N*) and by direct stimulation of the muscle (*M*). The first series were taken when sucrose 0·4% was added to the bath, later at the second arrow glucose 0·2% replaced the sucrose and at the third arrow the glucose was removed. Neon stimulator—rate of stimulation 4 per min.

In preparations which do not show the effect, it may be produced by first taking steps to reduce the glycogen of the preparation, such as stimulating it in glucose-free Tyrode solution for several hours until depression occurs. Glucose is then added to the bath for a few minutes to cause a temporary recovery, but its withdrawal now causes a further augmentation of the muscle responses (Fig. 2). Augmentation of the nerve-muscle responses occurs also and sometimes even to a greater extent when instead of replacing the 0·2% glucose containing Tyrode with glucose-free Tyrode solution, one containing 0·4% sucrose in order to keep the osmotic pressure constant (Fig. 3), is used.

If the nerve and muscle are stimulated alternately for short periods it can be shown that removal of the glucose affects both the responses, but augmentation may appear to nerve stimulation before it appears to direct muscle stimulation although admittedly such direct stimulation must involve the nerve elements

in the muscle. The augmentary effect of removal of glucose on the nerve-muscle responses appears to depend on a mechanism which is exhaustible, for it disappears after repeated changes of the bath fluid, and so far it has not been found possible to cause the effect to return by adding substances to the bath fluid. When on repeated replacement of the bath fluid the augmentary effect of glucose withdrawal has disappeared, there are not necessarily other signs of deterioration detectable in the preparation.

Fig. 3. Shows similar effects on another preparation which had been more exhausted by prolonged use. The relative effects of sucrose, glucose and glucose lack are seen. Stimuli duration: 500 μsec., 5 V., frequency 6 per min. At A, 0·4% sucrose in Tyrode solution; at B and D, 0·2% glucose; at C, glucose-free Tyrode solution.

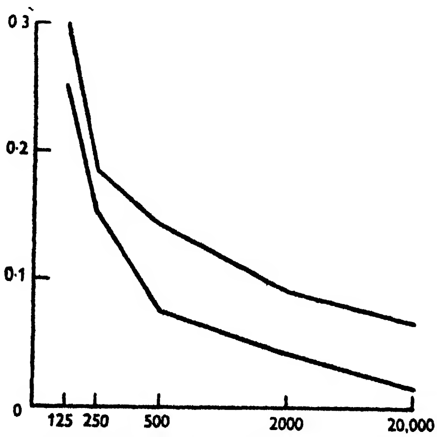


Fig. 4. The effect of glucose on the strength duration curves. The lower curve is that without glucose.

The augmented nerve-muscle response is associated with an increase in excitability of the preparation and is due at least in part to repetitive re-excitation of the muscle fibres. The increased excitability is seen when strength-duration curves are made. In the presence of glucose the curve lies above that obtained from the same preparation in the absence of glucose (Fig. 4). Removal of the glucose from the bath fluid causes the chronaxie of the preparation, when stimulated through its nerve, to be reduced to about a half. The effect is

similar to, but not as great as, that obtained on the addition of eserine to the bath fluid. The increase in excitability occurs on removal of glucose only if it produces also an augmentary nerve-muscle response.

It has long been known that lack of calcium increases the excitability of muscle and nerve and later abolishes neuromuscular transmission. Calcium also depresses the synthesis of acetylcholine (Feldberg, 1944–5). The calcium concentration of the normal Tyrode solution is 0·02 %. It was reduced to 0·01 or 0·005 % which is nearer the normal ionized calcium concentration in the blood, which is less than 0·005 %. In such a bath fluid the diaphragm preparation retains neuromuscular transmission but becomes abnormally sensitive to glucose lack (Fig. 5). In some preparations, however, the effect of calcium lack

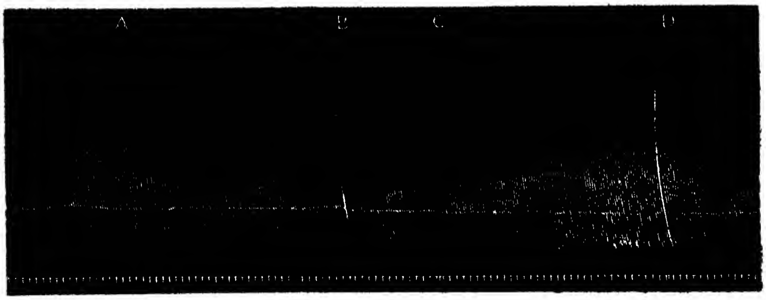


Fig. 5. Effect of reduced calcium on response of glucose withdrawal. At A, normal Tyrode solution containing 0·02 % calcium chloride was used; at B, modified Tyrode solution containing 0·01 % calcium chloride; at C, modified Tyrode solution without glucose; and at D, glucose was added without other change of fluid.

appears with even 0·01 $CaCl_2$ as shown by a partial failure of neuromuscular transmission. In such preparations the augmentation of the glucose withdrawal effect is absent. Particularly striking results, however, are obtained if the removal of glucose and the reduction of calcium are carried out simultaneously (Fig. 6).

If the diaphragm preparation is kept in glucose-free Tyrode solution with reduced Ca-concentration for longer periods, two other effects sometimes occur: a contracture and spontaneous twitchings. Contracture appears in the absence and in the presence of nerve stimulation. If it occurs during electrical nerve stimulation the contractions produced by the stimulation become reduced. It is thus a true contracture like that produced by acetylcholine on frog muscle or on denervated mammalian muscle. The contracture can be removed by adding glucose to the bath.

Denervation of the diaphragm 2 weeks previously completely abolished the augmentary effects of the lack of glucose and so does a full dose of curare. If, however, a dose of curare is added which is just sufficient to abolish the nerve response, in the presence of glucose, withdrawal of the latter has a decurarizing

effect; also, a larger dose of curare is necessary to abolish the response to nerve stimulation if glucose is absent than if it is present.

When the single augmented contraction obtained on nerve stimulation after removal of the glucose is analysed it becomes évident that the main effect is

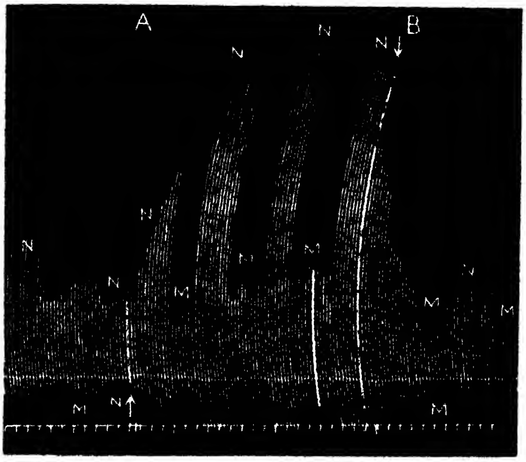


Fig. 6. Effects of simultaneous withdrawal of calcium and glucose. At A, Tyrode solution with 0·2% glucose replaced by solution containing 0·005% calcium and no glucose; at B, fresh solution of original calcium and glucose content.

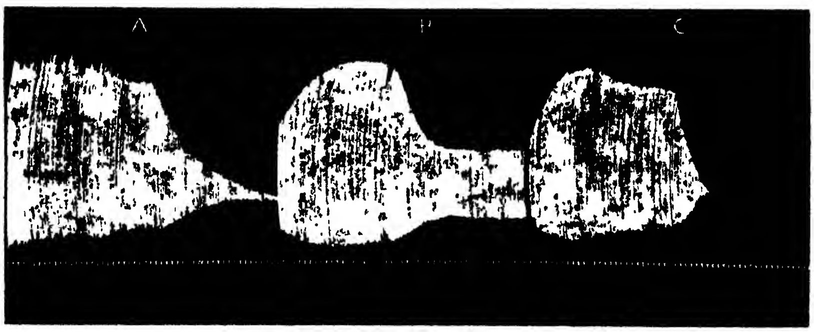


Fig. 7. Shows the effect of curare (30 μg.) in Tyrode solution containing in A, 0·02% glucose; B, containing no glucose; and C, 0·2% glucose.

concerned with the period of relaxation. McDowall & Shafei (1947) have pointed out that a delayed relaxation resembling that seen after eserine can be obtained when a preparation has been kept without stimulation for a few minutes. The first stimulus after such a period of rest produces a contraction with delayed relaxation, which is due to repetitive excitation of the muscle fibres. On withdrawal of glucose from the bath this delayed relaxation is more pronounced, and electrical records taken during this condition, by leading off from muscle to tendon, show an increase in the main action potentials of the muscle and

a more pronounced repetitive excitation of the muscle. The addition of small amounts of eserine to the Tyrode solution or a reduction of its calcium concentration produced the augmentary response on withdrawal of glucose in an exaggerated form. The concentrations of eserine used for this purpose must be small, about 1 μg. to the 50 ml. bath, otherwise the effect of the augmentary action of eserine interferes with that produced by the withdrawal of glucose. The synergistic action of these two changes, however, is evident from the following results: (*a*) in the absence of glucose from the bath fluid small doses of eserine exert a much stronger augmentary effect than when the fluid contains 0·02 % glucose as well (Fig. 8); (*b*) in preparations in which the withdrawal

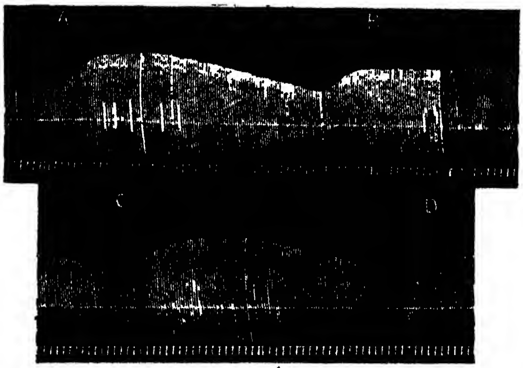


Fig. 8. The effect of the same dose of eserine at A, B, C and D, but without glucose at A and C, and with 0·2 % glucose at B and D.

of glucose produced no augmented nerve-muscle response it appeared when the experiment was repeated in the presence of eserine; and (*c*) the augmentary effect of eserine obtained in the absence of glucose is depressed when 0·2 % glucose is added to the fluid (Fig. 8).

The synthesis of acetylcholine by minced diaphragm is also reduced by glucose. Many workers have now shown that acetylcholine may be synthesized in vitro by slices of brain tissue (e.g. Quastel, Tennenbaum & Wheatley, 1936), by minced brain (Steadman & Steadman, 1939), and by ground nervous tissue (Feldberg, 1944–5). It has also been demonstrated that the synthesis is augmented by small amounts of glucose but is inhibited by larger amounts and by calcium (see Feldberg, 1944–5), although the former may not exceed that normally present in the blood. In view of the results just given on the effects of glucose on the contraction of the rat diaphragm, it appeared desirable to study how far glucose could affect the synthesis of acetylcholine by muscle. The method adopted was used by Feldberg (1944–5). This consists of incubating equal weights of ground-up muscle with and without glucose for 90 min. at 37° C. in eserinized saline and testing the potency of filtrates on eserinized frog's rectus. Ground muscle gave better results than minced muscle and it

was found that the synthesis continued for as long as 3 hr. The results obtained were the same in all experiments, namely, that glucose in amounts comparable with those which inhibit contractions or which occur in human blood reduced the synthesis of acetylcholine to less than one-tenth; for example from 5 to 0·5 μg./g. tissue.

DISCUSSION

It has been shown that the rat phrenic-diaphragm preparation is extremely sensitive to concentrations of glucose. The depression of its contractions when placed in a glucose-free solution is no doubt due to lack of a source of energy, the actual time of onset of the depression depending on the store in the preparation at the commencement of the experiment, and when this was considerable no depression might occur for several hours.* It has already been pointed out by Lee, Guenther & Meleney (1916) that the diaphragm contains more glycogen than any other muscle examined and is therefore remarkably resistant to fatigue. We are familiar with the necessity for glucose in regard to the isolated heart and intestine.

The augmentation of contraction which may precede the depression and last for a considerable time appears to be dependent on the nervous elements of the preparation, for it cannot be obtained in a denervated or fully curarized diaphragm. The results suggest that the augmentation is dependent on an increased synthesis of acetylcholine or its immediate precursor for the following reasons. Concentrations of glucose of the order of 0·2 % inhibit the synthesis of acetylcholine by a nerve-muscle preparation as they do synthesis by nervous tissue, it is thought, by taking up phosphorus (Feldberg, 1944–5). Withdrawal of glucose brings about increased contraction, the occurrence of delayed relaxation and repetitive responses by the muscle and the occurrence of twitches all of which are characteristic of the accumulation of acetylcholine produced by eserine. Withdrawal also reduces the effect of curare. The twitches produced are caused to disappear by those procedures which cause the disappearance of those produced by eserine. An increased excitability similar to that produced by eserine also occurs. The augmentation on withdrawing the glucose can readily be explained as a removal of an inhibition of the synthesis of acetylcholine, with the result that it accumulates as it does when its destruction by cholinesterase is prevented by eserine. It may be considered that the acetylcholine is synthesized and retained in such a way that it is ready for immediate release by the nerve impulse yet cannot be destroyed by any cholinesterase which may be present, possibly like the acetylcholine liberated by vasodilator nerves which is therefore not affected by atropine.

The effects of different rates and strengths of stimulation in producing depression of contraction of some muscle are of some interest. If the fibres

* It has now been shown that the depression is due to a neuromuscular block. This is analysed in a subsequent paper.

which are stimulated with weaker stimuli contract maximally, the absence of depression on withdrawal of glucose must mean that they acquire glucose from adjacent resting fibres, for if the stimulus is increased so as to include these fibres, depression rapidly ensues.

The acetylcholine may bring about its effects in two ways and there is evidence that both occur. (1) It may cause the muscle to contract more forcibly, and this is suggested by the production of augmentation when maximal stimuli of very short duration are used. (2) It may, in virtue of the increased acetylcholine released, stimulate an additional number of muscle fibres. This occurrence is indicated by the greater ease with which the augmentation occurs with submaximal stimuli and the appearance of delayed relaxation and the repetitive responses such as are produced by eserine with stimuli of short duration.

An alternative explanation might be that the acetylcholine sensitizes muscle fibres other than those stimulated so that they become excited by the action potentials of the fibres stimulated. It might be considered that this view is supported by the finding of the increased excitability unless it can be held that the increased excitability is dependent on the liberation of a stimulating amount of acetylcholine by an otherwise subminimal stimulus.

Whatever the exact nature of the action of the acetylcholine, it is clear that its peripheral synthesis is related to the glucose in the preparation, and thereby to the energy mechanism of the muscle. The conclusion also follows that the effect of any given stimulus is determined by the amount of acetylcholine available to be liberated at the nerve endings, a fact which may be important in all questions concerning the work done in response to a given stimulus, especially in regard to fatigue.

SUMMARY

The contractions of the phrenic-diaphragm preparation of the rat are commonly augmented before being depressed by lack of glucose. The depression is considered to be due to lack of energy, but the augmentation which is associated with increased excitability is thought to be due to a peripheral accumulation of acetylcholine as a result of increased synthesis. Glucose also inhibits the synthesis of acetylcholine by ground-up muscle.

The authors are indebted to Dr J. A. C. Knox who was good enough to make records of the electrical responses, and to Dr Hajdu who has since considerably extended the observations. This will be the subject of a separate paper.

REFERENCES

Bülbring, E. (1946). *Brit. J. Pharmacol.* **1**, 38.
Brown, G. L. & Burns, B. D. (1947). Private communication.
Feldberg, W. (1944–5). *J. Physiol.* **103**, 367.
Lee, F. S., Guenther, A. E. & Meloney, H. E. (1916). *Amer. J. Physiol.* **40**, 446.
McDowall, R. J. S. & Shafei, A. Z. (1947). *J. Physiol.* **106**, 2*P*.
Quastel, J. H., Tennenbaum, M. & Wheatley, H. M. (1936). *Biochem. J.* **30**, 1668.
Steadman, E. & Steadman, E. (1939). *Biochem. J.* **33**, 811.

J. Physiol. (1949) 108, 33–36 612.851

THE MEASUREMENT OF THE TEMPERATURE AT THE EARDRUM DURING THE CALORIC TEST OF THE LABYRINTH

By J. J. GROEN and L. B. W. JONGKEES

From the Clinic for Ear, Nose and Throat Diseases, University of Utrecht

(*Received* 8 *January* 1948)

The caloric test is often performed with water at temperatures, equidistant from 37° C., in order to produce equal stimulations of the labyrinth (Hallpike, 1943). The effect of hot caloric stimulation, however, is generally smaller than that of cold stimulation. This phenomenon may be due to different causes. The heat-loss of hot water in the external auditory canal may be larger than the heat-gain of cold water, with the result that the stimuli are no longer equal at the drum; on the other hand, the phenomenon may be the result of some vascular influence. To investigate this problem we followed the method indicated by Jongbloed & Noyons (1941).

METHOD

The temperature was measured electrically with a thermopoint, while the indifferent point was placed in a thermoregulator at 37° C. The metals used for the thermocouple were manganine and constantan fibre. In the first tests the thermopoints were enclosed in a very thin glass tube rounded at the end, but on account of its fragility this was too dangerous. We therefore employed collodion in many layers, allowing plenty of time for each layer to dry. About 40 layers were used.

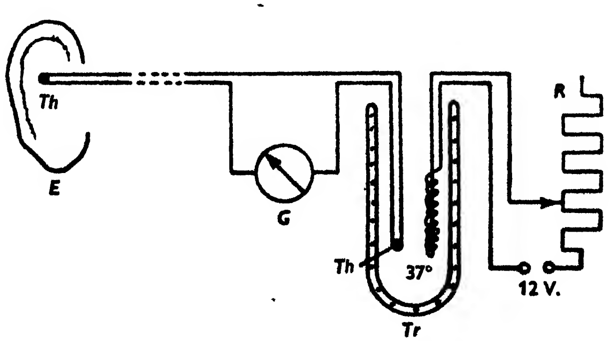


Fig. 1. Diagram of arrangement for the thermoelectrical measurement of the temperature in the external auditory canal. *Th* = thermopoint, *G* = galvanometer, *E* = ear, *Tr* = thermoregulator, *R* = resistance. The thermocouple is made of manganine and constantan fibre.

This made the tube pliant so that the external auditory canal or drum could not be damaged. The time of indication of the system is about 1 sec. The experiments were performed on normal men. One thermopoint was placed against the drum by the subject himself under control of the observer. This is not difficult after practice.

RESULTS

The temperature of the external auditory canal is 0·4° C. lower than the rectal temperature on the average, though it may sometimes be higher (e.g. in a subject suffering slightly from influenza the temperature of the ear was 38·4° C. and that of the rectum 37·8° C.). In twenty-five subjects the average temperature at the drum was 36·8° C. All these temperatures were measured in a moist external auditory canal. Even if the auditory canal is closed with a piece of cotton-wool the observed temperature is lower in a dry than in a moist canal.

TABLE 1. Average values of a number of tests in different subjects

Stimulating temperature (° C.)	Limiting temperatures of drum (ml. water)		
	5	50	500
44	40·5	40·9	42·5
43	40·2	—	—
41	39·1	—	—
40	38·7	39·7	—
38	37·7	37·8	—
36	35·8	35·8	—
34	34·5	34·4	—
32	33·4	32·8	—
30	31·8	31·5	30·5
15	20·0	19·2	17·1

In the following experiments we measured the temperature during the performance of the caloric test, observing the nystagmus at the same time. During the caloric test the temperature of the external auditory canal approaches its maximum in about 10 sec. and then returns slowly to the original value. The duration of the change of temperature is of the same order as the duration of the nystagmus. In some instances, however, the cold water reactions last longer than the drop in temperature. The influence of the temperature and of the quantity of the stimulating water on the maximal deviation of the temperature of the ear is indicated in Table 1.

DISCUSSION

The results indicate that in the caloric test the temperature of the drum is influenced by the quantity of water. The influence of the quantity of water is of very small significance for the duration of the nystagmus (Jongkees, 1947); on the other hand, the influence of the temperature of the water, though not very large, cannot be neglected.

As different quantities of water produce different temperatures at the drum it seems probable that the total amount of heat added or subtracted, rather than the temperature of the water, is the most important factor. To calculate

the effect of a certain quantity of heat is, however, difficult because the vestibular reaction is the result of a difference in temperature of various parts of the labyrinth. A flux of heat passing through the petrous bone, as in the caloric test, will reach not only the lateral wall but also the more medial parts, though at a later time. The vestibular reaction is dependent on the temperature of the different parts of the labyrinth and not only on the total amount of heat brought into the external auditory canal. This explains the behaviour of the caloric reaction, which does not seem to be in accordance with expectations.

An example may be given to illustrate the influence of the quantities of water on the temperature of the drum and on the duration of the nystagmus. In a normal subject the reaction of the left ear with water of 30° C. was as follows:

Duration of the nystagmus after syringing with 5 ml.	105 sec.
Minimal temperature at the drum	32·0° C.
After syringing with 50 ml. duration of the nystagmus	105 sec.
Minimal temperature	30·5° C.

With water of 44° C. the same pattern was found:

Syringing with 5 ml. gives a duration of the nystagmus	80 sec.
Maximal temperature	40·5° C.
After the use of 50 ml. duration of the nystagmus	80 sec.
Maximal temperature	41·5° C.
With 500 ml. the duration of the nystagmus	90 sec.
Maximal temperature	43·0° C.

In many cases a cold reaction (i.e. a heterolateral nystagmus) was caused by water of 38° or even 39° C. (Jongkees, 1948). This is not the result of lowering of temperature at the drum through the cooling of the water. An example may illustrate this. In a normal subject the following values were found: syringing of the right ear with 50 ml. water at 40° C. gives homolateral nystagmus at a drum temperature 38·1° C.; 38° C. gives heterolateral nystagmus at a drum temperature 37·2° C.; 36° C. gives heterolateral nystagmus at a drum temperature 36·2° C. The temperature of the drum before the tests was 36·6° C. It is apparent that a heterolateral nystagmus, i.e. a cold reaction, was elicited by water which caused a rise of temperature at the eardrum.

In one case we were able to perform the caloric test in normal conditions, with a temperature of the auditory canal at 36·5° C., while some days later, when the patient had a fever, the temperature of the auditory canal was 38° C. In both instances the test with water of 30° and 44° C. gave identical durations of the nystagmus within normal limits. Finally it is to be noted that in one subject the temperatures of the two auditory canals may differ by as much as 0·5° C.

SUMMARY

1. The temperature of the external auditory canal as measured with a thermocouple appears to be about 0·4° C. below the rectal temperature. The temperatures of the two ears may differ by 0·5° C.

2. Water temperatures of 30° and 44° C. used in the caloric test produce almost equal deviations of the temperature at the drum.

3. Heterolateral nystagmus may be caused by an increase of temperature at the eardrum in the normal position of the subject.

4. Different quantities of water which give similar results in the caloric test nevertheless cause different temperatures at the drum. This may be explained by a difference in dispersion of the flux of heat in the petrous bone.

REFERENCES

Hallpike, C. S. (1943). *J. Laryng.* **58**, 349.

Jongbloed, J. & Noyons, A. K. M. (1941). *Ned. Tijdschr. Geneesk.* p. 2995.

Jongkees, L. B. W. (1947). *Ned. K.N.O. Vereeniging,* Meeting 23 May.

Jongkees, L. B. W. (1948). *Arch. oto-laryng.* In the Press.

J. Physiol. (1949) 108, 37–77 612.813

THE EFFECT OF SODIUM IONS ON THE ELECTRICAL ACTIVITY OF THE GIANT AXON OF THE SQUID

By A. L. HODGKIN and B. KATZ

From the Laboratory of the Marine Biological Association, Plymouth, and the Physiological Laboratory, University of Cambridge

(*Received* 15 *January* 1948)

Experiments with internal electrodes suggest that the active reaction of nerve is not a simple depolarization of the kind postulated by Bernstein (1912) and Lillie (1923). In the giant axon of the squid, the resting membrane potential appears to be about 50 mV. whereas the action potential is of the order of 100 mV. (Curtis & Cole, 1942; Hodgkin and Huxley, 1939, 1945). This result implies that the surface membrane undergoes a transient reversal of potential difference during the passage of the nervous impulse. The magnitude of the reversal cannot be measured precisely, because of uncertainties concerning the liquid junction potential between the axoplasm and the internal recording electrode. But there is now little doubt that the membrane potential of certain types of nerve fibre does undergo an apparent reversal which cannot be reconciled with the classical form of the membrane theory. Several attempts have been made to provide a theoretical basis for this result (Curtis & Cole, 1942; Hodgkin & Huxley, 1945; Höber, 1946; Grundfest, 1947), but the explanations so far advanced are speculative and suffer from the disadvantage that they are not easily subject to experimental test. A simpler type of hypothesis has recently been worked out, in collaboration with Mr Huxley, and forms the theoretical background of this paper. The hypothesis is based upon a comparison of the ionic composition of the axoplasm of a squid nerve with that of the sea water in which experimental preparations are normally immersed. The potassium concentration of fresh squid axoplasm appears to be some twenty to forty times greater than that in sea water, whereas the sodium and chloride ions may be present in concentrations which are less than one-tenth of those in sea water (Steinbach, 1941; Steinbach & Spiegelman, 1943). The resting membrane potential is supposed to arise in a manner which is essentially similar to that postulated in Bernstein's form of the membrane theory. The resting membrane is assumed to be permeable to potassium and

possibly to chloride ions, but is only very sparingly permeable to sodium. There should, therefore, be a potential difference of the correct sign and magnitude across the surface membrane of a resting nerve fibre.

According to the membrane theory excitation leads to a loss of the normal selectively permeable character of the membrane, with the result that the resting potential falls towards zero during activity. This aspect of the theory is at variance with modern observations and must be rejected. However, a large reversal of membrane potential can be obtained if it is assumed that the active membrane does not lose its selective permeability, but reverses the resting conditions by becoming highly and specifically permeable to sodium. The reversed potential difference which could be obtained by a mechanism of this kind might be as great as 60 mV. in a nerve with an internal sodium concentration equal to one-tenth of that outside. The essential point in the hypothesis is that the permeability to sodium must rise to a value which is much higher than that to potassium and chloride. Unless this occurs the potential difference which should arise from the sodium concentration difference would be abolished by the contributions of potassium and chloride ions to the membrane potential. The hypothesis therefore presupposes the existence of a special mechanism which allows sodium ions to traverse the active membrane at a much higher rate than either potassium or chloride ions.

A simple consequence of the hypothesis is that the magnitude of the action potential should be greatly influenced by the concentration of sodium in the external fluid. Thus the active membrane should no longer be capable of giving a reversed e.m.f. if the external sodium concentration were made equal to the internal concentration. On the other hand, an increase of membrane reversal would occur if the external sodium concentration could be raised without damaging the axon by osmotic effects. Experiments of this kind are difficult to make when external electrodes are used for recording; for the sodium content of the external medium cannot be varied without changing the electrical resistance of the extra-cellular fluid, and this would in itself cause a large alteration in the magnitude of the recorded action potential. We have therefore studied the influence of sodium concentration on the form and size of the action potential recorded with an internal electrode in the giant axon of the squid.

APPARATUS

The general plan of the equipment was essentially similar to that used by Hodgkin & Huxley (1945) and need not be described in detail. A diagram of the recording cell is shown (Fig. 1) in order to facilitate description of the experimental procedure. The walls of the cell were made of glass or Perspex. The Perspex was at first coated with a thin film of paraffin wax, but no adverse effects were observed when this precaution was omitted. The cell was illuminated from the side and this had the advantage that a double image of the axon could be obtained in the microscope by using a single mirror instead of the more complicated arrangement employed by Hodgkin & Huxley (1945). The mirror was removed from the cell as soon as the microelectrode had been inserted to the correct distance.

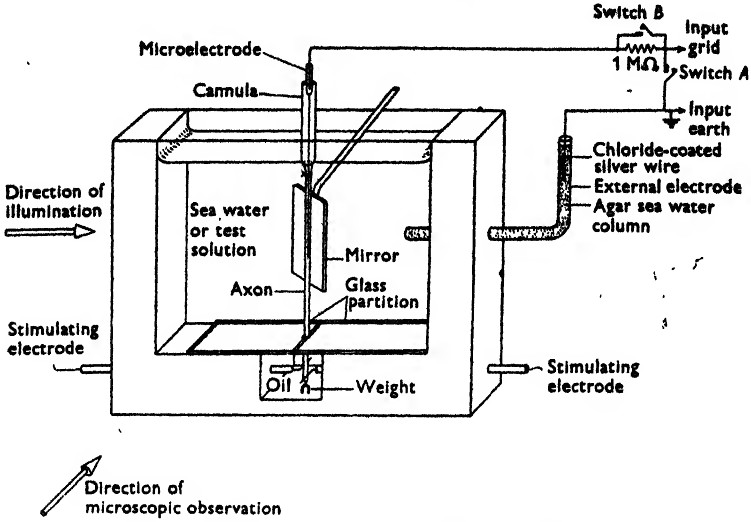


Fig. 1. Simplified diagram of recording cell.

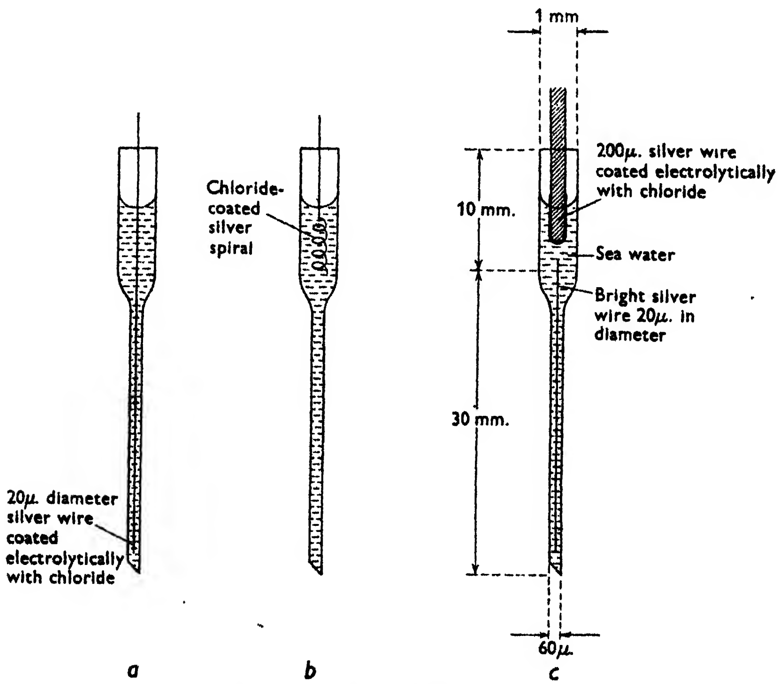


Fig. 2. Diagrams of microelectrodes: *a* and *b*, types formerly employed; *c*, type used in present work.

Microelectrode. The microelectrodes employed in earlier work (Hodgkin & Huxley, 1945) are shown in Fig. 2*a* and *b*. Type *a* had a reasonably low resistance, but it was unsuitable for prolonged experiments because the electrode potential was unsteady. The electrode therefore had to be withdrawn from the fibre at frequent intervals in order to allow its potential to be measured against that of the external electrode. Type *b* gave a steadier potential, but its resistance was so high that action potentials were reduced in magnitude by the stray capacity of the input circuit. The electrode used in the present work is shown in Fig. 2*c* and was designed to combine the advantages of both the first and second types. A relatively thick silver wire made contact with the sea water in the shank of the tube, and was electrolytically coated with chloride. In addition, a bright silver wire was thrust down the capillary to within 1·5 mm. of the tip. When a steady potential was measured this electrode acted in the same way as the second type of electrode. The bright silver wire did not influence the steady potential, because it was effectively shunted by the column of sea water in the capillary. Under these conditions the electrode resistance was determined by the whole length of the column of sea water which amounted to several megohms. However, this resistance did not affect the measurements, because the input resistance of the amplifier was greater than $10^9\ \Omega$. When a transient potential difference such as a spike was measured, the column of sea water was momentarily short-circuited by the polarizable silver wire which acted like a condenser connected between the tip and base of the microelectrode. The electrode therefore behaved as though it had a relatively low resistance and thus avoided the errors which would otherwise have been introduced by stray capacity. It can be shown theoretically that an electrode of this kind should not introduce any distortion of the action potential, provided that the polarization capacity of the bright silver wire is large compared to the stray capacity of the input circuit. But these calculations need not be presented, since the performance of the electrode could be tested directly in a manner which will now be described.

The first test consisted in the sudden application of a potential difference to the tip of the microelectrode. A rectangular wave of current was passed through a 5 kΩ. resistance connected between the external electrode of the recording cell and earth. The terminal of the external electrode was connected directly to the input stage, and the resulting deflexion of the cathode ray tube recorded photographically. This test showed that the amplifier and input stage operated with an exponential lag of 4 μsec. The tip of the microelectrode was then lowered into the sea water, the input lead transferred from the external electrode to the microelectrode and a second photograph obtained. The two records were found to differ by less than 5%, thus demonstrating that the total lag of the recording system was of the order of 4 μsec. This lag may be neglected with safety, since the rising phase of the action potential occupied about 200 μsec.

A test which was essentially similar to this was also made using rectangular steps of current lasting many seconds, and it was again found that the microelectrode introduced no perceptible change in the size or shape of the potential recorded.

Amplifier and recording system. The characteristics of the d.c. amplifier and input stage were essentially similar to those described by Hodgkin & Huxley (1945), as were the methods of calibration employed. Errors which might have arisen from non-linearity or cathode-ray tube curvature were eliminated by comparing the records directly with a calibration grid which was obtained by photographing the series of oscillograph lines resulting from successive application of 10 mV. steps of potential to the input.

Records of the rate of change of membrane voltage during the action potential were obtained by electrical differentiation. This was achieved by introducing a single stage of condenser coupling into the d.c. amplifier. The time constant of the condenser and resistance used for differentiating was approximately 13 μsec. Under these conditions the output of the amplifier should be proportional to the rate of change of the input. What is measured is not exactly equal to the instantaneous rate of change at any given moment, but is more nearly equivalent to the average rate of change over a period which is reasonably short compared to the action potential. The rate circuit and amplifier were tested with rectangular inputs or sine waves of known frequency and amplitude. The rate amplifier was calibrated by comparing the absolute magnitude of the action potential in millivolts with the values obtained by graphical integration of the first

phase of the rate record. The quotient of the two quantities gives the scale factor for the rate amplifier.

Rates were occasionally measured by graphical differentiation of the action potential. This procedure was laborious, and subject to considerable uncertainty unless the action potential was recorded on an expanded time-scale. The difference between electrical and graphical measurements usually amounted to about 10%, and there may be an error of 10% or even more in the absolute magnitude of the rates quoted in this paper. For the most part we shall be concerned not with absolute rates but with the relative magnitude of the rates in solutions of varying sodium concentration, and the error here is likely to be less than 5%.

Artificial solutions. Test solutions were usually made by mixing isotonic solutions in different proportions. The values used in preparing isotonic solutions are given in Table 1 and were based on cryoscopic data in International Critical Tables. Concentrations were chosen to give a freezing-point depression of 1·88° C. which appears to be the correct value for sea water of salinity 3·45% (Glazebrook, 1923). No cryoscopic data could be obtained for choline chloride and the figure of 0·6 M must be regarded as a guess.

Sea water was used as a normal medium, since no Ringer's solution applicable to the squid has yet been developed. An artificial sea-water solution was also employed on certain occasions, and was made according to the formula in Table 1. No appreciable difference could be detected between the action of this solution and that of sea water.

Sodium-rich solutions were made by adding solid sodium chloride to sea water.

Tests with indicators showed that all solutions employed had approximately the same pH as that of sea water.

Dextrose solutions were made up at frequent intervals and were stored in a refrigerator when not in use.

TABLE 1. Composition of stock solutions

I. Isotonic solutions

Solution	Solute	Molality	g./1000 g. H_2O
a	NaCl	0·56	32·7
b	KCl	0·56	41·8
c	$CaCl_2$	0·38	42·1
d	$MgCl_2$	0·36	34·3
e	$NaHCO_3$	0·56	47·1
f	Dextrose	0·98	177·0
g	Sucrose	0·93	319·0
h	Choline chloride	0·60	83·6

II. Artificial sea water

804 parts *a*:18 parts *b*:28 parts *c*:146 parts *d*:4·6 parts *e*.

Liquid junction potentials in the external circuit. The action of a test solution was examined by sucking out the sea water from the recording cell and running in a new solution. The test solution did not alter the potential of the external electrode, since the silver chloride surface was separated from the recording cell by a long column of agar sea water. However, the test solution set up a small liquid junction potential at the edge of the agar sea-water column and this had to be measured before the effect of a test solution on the resting potential could be evaluated.

Junction potentials were measured by dipping a silver chloride electrode (in some cases the microelectrode itself) into a beaker filled with sea water which was connected to the recording cell by means of a saturated KCl bridge. The system employed was, therefore,

Ag.AgCl	Sea water	Saturated KCl	Test solution in recording cell	Agar sea-water column in external electrode	AgCl.Ag

In measuring junction potentials an attempt was made to reproduce, as far as possible, the experimental conditions used in examining a living nerve. The data obtained are, therefore, not strictly comparable to those given by standard physicochemical methods, but should provide the

right corrections for the present research. The saturated KCl bridge method is known to be unsatisfactory in certain respects, but it probably gives results of an accuracy sufficient for the present purpose.

The results obtained are shown in Table 2, and give the corrections which have to be subtracted from any apparent change in resting potential produced by the solution in question. No value is given for isotonic dextrose, since this solution gave an unsteady potential which increased with time to a large value.

Liquid junction potentials for solutions of intermediate strength (e.g. 0·7 sea water, 0·3 dextrose) were obtained by interpolation.

TABLE 2. Liquid junction potentials measured by saturated KCl bridge method

Test solution	E sea water − E test solution (mV.)
1 part sea water: 1 part isotonic dextrose	+2·6
1 part sea water: 4 parts isotonic dextrose	+6·0
1000 c.c. sea water + 15 g. NaCl	−0·7
1 part sea water: 1 part 0·6 M-choline chloride	−0·7

Experimental procedure. Giant axons, with a diameter of 500–700 μ., were obtained from the hindmost stellar nerve of *Loligo forbesi*. The methods of mounting the axon and of inserting the microelectrode require no description, since they were essentially similar to those employed by Hodgkin & Huxley (1945). Before introducing the microelectrode, a value was obtained for the small potential difference between the microelectrode and the external recording electrode. The potential difference was obtained by dipping the microelectrode into the sea water in the recording cell (which was normally connected to earth by the external electrode) and comparing the position of the base-line with the value obtained by 'earthing' the input lead. The potential difference between the two electrodes usually amounted to several millivolts and this value had to be subtracted from the apparent resting potential. Errors which might have arisen from amplifier drift were avoided by repeated checks of the amplifier zero, but this procedure did not obviate errors caused by changes in the microelectrode potential. The microelectrode could not be withdrawn during the course of an experiment, and we therefore had to rely on the stability of its potential. In the most complete experiments the electrode did not drift by more than 4 mV. in about 4 hr., but changes equivalent to 2 mV./hr. were sometimes encountered. We attempted to allow for changes in microelectrode potential by a sliding correction, but measurements of the resting potential cannot be presented with the same confidence as can those relating to the amplitude of the spike. The method of obtaining the amplifier zero requires comment since this was not such a simple operation as might at first be supposed. In the interests of stability it was desirable, first, that the input circuit should never be open-circuited; and secondly, that the nerve membrane should never be short-circuited. The following procedure was therefore adopted. First, a photographic record of the action potential and resting base-line was obtained with switch *A* open and switch *B* closed (Fig. 1). Switch *B* was opened then switch *A* closed and a second record obtained. This operation gave the amplifier zero but did not short-circuit the membrane, since this was protected first by the resistance of the microelectrode and secondly by the 1 MΩ. resistance. The switching procedure was reversed when the amplifier zero had been obtained. In a few experiments, switch *B* was left open throughout. This increased the recording lag from 4 μsec. to 11 μsec., but it did not cause any measurable change in the form of the action potential or its derivative.

The giant fibre was normally stimulated at 40 per min. throughout the entire period of experimental test.

RESULTS

Electrical properties of axons immersed in sea water

The magnitudes of the action potential, resting potential and positive phase were measured as a matter of routine at the beginning of each experiment, and are shown in Table 3. Approximate values for the maximum rates of rise and fall of the spike are also included. A few axons gave spikes less than 80 mV., but were not used for quantitative measurements because they deteriorated rapidly. The values for spike height are in good agreement with those obtained by Hodgkin & Huxley (1945), but are considerably smaller than those reported by Curtis & Cole (1942). The average value for the resting potential (48 mV.) is slightly smaller than that given by Curtis & Cole (51 mV.), but a difference of this kind is to be expected since Curtis & Cole used KCl in the micro-electrode, whereas we employed sea water. The average action potential was about 20 mV. smaller than that given by Curtis & Cole. But a more serious discrepancy arises from the fact that we have never observed action potentials greater than 100 mV. at 18–23° C., whereas Curtis & Cole describe a spike as large as 168 mV. in a fibre which gave a resting potential of 58 mV. The matter is not one that can be lightly dismissed, because the existence of a fibre capable of giving an overshoot of 110 mV. has far-reaching implications. We are no longer inclined to think that our relatively small action potentials can be attributed to the poor condition of the experimental animals, since a number of the squids employed were extremely lively and in perfect condition. Nor does it seem likely that axons were damaged in the process of isolation, since micro-electrodes were sometimes inserted into axons which were still surrounded by

TABLE 3. Electrical properties of axons in sea water

	Temperature (° C.)	Resting potential (mV.)	Action potential (mV.)	Membrane reversal (mV.)	Positive phase (mV.)	Maximum rate rise (V.sec.$^{-1}$)	Maximum rate fall (V.sec.$^{-1}$)
	22	46	85	39	14	—	—
	20	52	93	39	11	—	—
	21	52	86	34	10	490	290
	24	51	83	32	13	580	380
	22	50	86	36	15	650	400
	22	49	93	44	15	770	460
	20	40	87	47	15	560	330
	20	51	98	47	15	630	390
	—	48	87	39	15	520	340
	21	46	89	43	16	600	360
	20	53	99	46	14	1000*	530*
	20	46	85	39	14	620	480
	19	42	85	43	15	490	330
	20	45	86	41	16	590	360
	21	45	82	37	15	680*	350*
Average	21	48	88	40	14	630	380

* Indicates that these values were obtained by graphical differentiation. The values for resting potential are those observed with a microelectrode containing sea water. No correction has been made for the junction potential between axoplasm and sea water.

a greater part of the nerve trunk and had been subjected to a minimum amount of dissection. Hodgkin & Huxley's (1945) experiments indicate that the process of inserting a microelectrode did not in itself reduce the action potential, so that the possibility of damage at this stage may also be reasonably dismissed. Curtis & Cole's experiments may have been made at a different temperature, but this does not account for the discrepancy, since the action potential increases by 5–10 % when the nerve is cooled from 20 to 0° C. and decreases as the temperature is raised above 20° C. (unpublished results). Apart from the possibility of instrumental error, the only explanation which can be offered is that there is a real difference between the properties of *L. peali* used at Woods Hole and *L. forbesi* used at Plymouth.

Sodium-free solutions

Many years ago Overton (1902) demonstrated that frog muscles became inexcitable when they were immersed in isotonic solutions containing less than 10 % of the normal sodium-chloride concentration. He also showed that chloride ions were not an essential constituent of Ringer's solution, since excitability was maintained in solutions of sodium nitrate, bromide, sulphate, phosphate, bicarbonate, benzoate, etc. On the other hand, lithium was found to be the only kation which provided a reasonably effective substitute for sodium. Overton was unable to repeat his experiment with a frog's sciatic nerve, which maintained its excitability for long periods of time in salt-free solutions. But it now seems likely that this result was due to retention of salt in the interstitial spaces of the nerve trunk. Thus Kato (1936) found that application of isotonic dextrose to single medullated fibres of the frog caused a rapid but reversible loss of excitability, and a similar result was obtained by Erlanger & Blair (1938) on the sensory rootlets of the bull-frog. Kato's result has also been confirmed by Huxley & Stämpfli (unpublished experiments), who applied both isotonic sucrose and isotonic dextrose to single medullated fibres of the frog and found that conduction is blocked reversibly within a few seconds when the saline content falls below about 0·011 M. Katz (1947) has shown that isotonic sucrose mixtures abolish the action potential of *Carcinus* axons if the sodium-chloride concentration is less than 10–15 % of that normally present in sea water. Further experiments on the effect of sodium-free solutions on *Carcinus* axons were made by one of us and will be summarized, because they provide a useful addition to the work with squid axons. In the first place, the action of isotonic dextrose on a single *Carcinus* axon is exceedingly rapid. The action potential is blocked in a few seconds and is restored in a similar space of time by restoration of saline. The speed at which these solutions act is not surprising, since solute molecules have to diffuse across a distance of only a few micra of loose connective tissue in order to reach the surface membrane of the axis cylinder. Further evidence can be obtained for the conclusion of Overton

(1902) and Lorente de Nó (1944, 1947) that it is the sodium and not the chloride ion which is essential for propagation. Thus axons are blocked by a mixture of 50 % isotonic choline chloride and 50 % dextrose, although this solution contains three or four times as much chloride as that present in a solution containing the minimum amount of sodium in the form of sodium chloride. The blocking effect of the first solution is not due to any harmful property of choline, since propagation occurs satisfactorily through a mixture of 50 % choline chloride

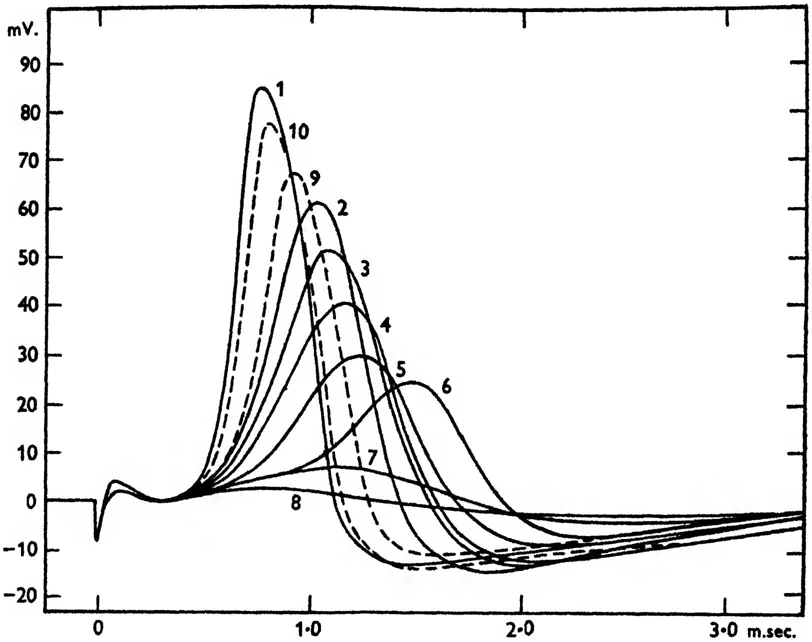


Fig. 3. Action of isotonic dextrose. Record 1: action potential in sea water just before application of dextrose. 2–8: records taken at following times after arbitrary zero, defined as moment of application of dextrose: 2, 30 sec.; 3, 46 sec.; 4, 62 sec.; 5, 86 sec.; 6, 102 sec.; 7, 107 sec.; 8, 118 sec. Record 9 taken 30 sec. after reapplication of sea water; 10, record at 90 and 500 sec. after reapplication of sea water (only one curve is drawn since the responses at these times were almost identical).

and 50 % sea water. Another point is that propagation is not affected by replacing the chloride in sea water with sulphate. All these experiments support the view that removal of sodium is the primary cause of block in salt-free solutions. A subsidiary factor may be the removal of calcium, since *Carcinus* axons do not survive for any length of time in a medium from which all traces of calcium have been removed.

The records in Fig. 3 show what happens to the membrane action potential when Overton's experiment is repeated on the giant axon of the squid. Curve 1 shows the electrical response of an axon immersed in sea water. Isotonic

dextrose was substituted for sea water as soon as this record had been obtained. The operation of changing solutions took 30–60 sec. and the zero time to which subsequent records are referred was defined at a somewhat arbitrary point during this process. Records 2–8 show how the action potential changed as the preparation came into diffusion equilibrium with the new medium. The spike amplitude dropped progressively and eventually fell to a very small value (records 7 and 8). This residual deflexion was almost certainly due to electrotonic spread from the part of the axon surrounded by oil which was not affected by the test solution. Removal of salt had a very striking effect on the rate of rise of the action potential which decreased to about 1/12 of its former amplitude after 107 sec. On the other hand, the rate of fall and the positive phase changed much less rapidly. The resting potential appeared to increase with time, but this effect may be attributed to the external liquid junction potential which could not be evaluated in this experiment; all records have therefore been traced from the same base-line. At 6 min. after zero, sea water was restored, with the result that the action potential recovered rapidly to a value which was close to that observed initially. The effect of isotonic dextrose thus appears to be almost completely reversible.

The action potential was also found to be abolished reversibly by a mixture of 50% isotonic dextrose and 50% choline chloride. Only one satisfactory experiment was performed, but this gave a result which was essentially similar to that in Fig. 3. On the other hand, the action potential was maintained at a value of about 70 mV. in a solution containing 50% isotonic dextrose and 50% sea water, or in one containing 50% choline chloride and 50% sea water. Axons from the squid therefore behave like those of *Carcinus*, in that a certain amount of external sodium is necessary for production of the action potential.

Fig. 3 shows that the action of isotonic dextrose was considerably slower in the giant axon of the squid than it was in axons from *Carcinus*. The difference is not surprising since *Carcinus* axons are surrounded by only 3 μ. of connective tissue, whereas the squid axons were rarely dissected cleanly and in this experiment the axon was left with a layer of tissue about 110 μ. in thickness. Such a thickness of external tissue is of the right order of magnitude to account for the delay in terms of diffusion. A detailed analysis of the process of equilibration has not been attempted, but a rough calculation suggests that the delay may reasonably be attributed to diffusion of sodium chloride from the adventitious tissue surrounding the axon into the large volume of isotonic dextrose in the recording cell. After the records in Fig. 3 had been obtained, the axon was immersed in a solution containing 20% sea water and 80% isotonic dextrose. In this solution the action potential fell rapidly to a value corresponding to that in record 5, and underwent only a small reduction during the subsequent period of 14 min. It therefore appears that the action of salt-

free solution was 80% complete in about 90 sec. This figure can be used to calculate an apparent diffusion constant if it is assumed that the fluid outside the preparation was completely stirred and that the diffusion process operated in the same manner as for a single substance diffusing into a slab of tissue 110 μ. in thickness. The value found for this experiment was about 0·1 cm.^{2}/day and values of this order of magnitude have been obtained in other cases. The diffusion constant for sodium chloride in water is 1·0 cm.^{2}/day and for dextrose 0·5 cm.^{2}/day (Landolt-Börnstein, 1931). The lag in the action of salt-free solutions can be explained if diffusion through the connective tissue and interstitial spaces in the remains of the nerve trunk are assumed to be about 1/7 of those in water. This is a reasonable assumption, since Stella (1928) concluded that diffusion of phosphate through the extra-cellular part of the frog's sartorius muscle was very slow compared to that in free solution. Another factor which may have retarded diffusion in the later stages is that the external solution was not stirred mechanically after the initial process of applying the test solution had been completed.

The effect of solutions of reduced sodium content on the resting potential and action potential

The general action of solutions containing a low sodium concentration is illustrated by Fig. 4. Record *a* 1 shows the action potential of an axon immersed in sea water. The base-line has been displaced from the zero of the calibration scale by an amount which corresponds to the resting potential. The zero was determined by short-circuiting the amplifier input and subtracting the small difference of potential which existed between the two recording electrodes. The zero therefore occurs at the potential which would have been observed if the microelectrode had been withdrawn and placed in the sea water outside the axon. Record *a* 2 shows the resting potential and action potential recorded after 16 min. in a solution containing 33% sea water and 67% isotonic dextrose. The method of obtaining the resting potential was similar to that formerly employed, except that an additional correction for the liquid junction potential has been introduced. The resting potential would have appeared to be 4·2 mV. larger if no such allowance had been made. The zero on the record now corresponds to the potential which would have been observed if the microelectrode had been withdrawn and connected to the solution in the recording cell by means of a saturated KCl bridge. Record *a* 3 was obtained 14 min. after replacing sea water in the recording cell. The action potential was 5 mV. less than that at the beginning of the experiment, but the difference was small compared to the decrease shown by *a* 2. The spike also arose with a greater delay, although the form and rate of rise were close to those observed originally. An effect of this kind is inevitable because the test solution diffused from the upper part of the recording cell into the region of nerve surrounded by oil. The

total conduction time was increased by this process, and the effect was only very slowly removed by application of sea water to the upper part of the

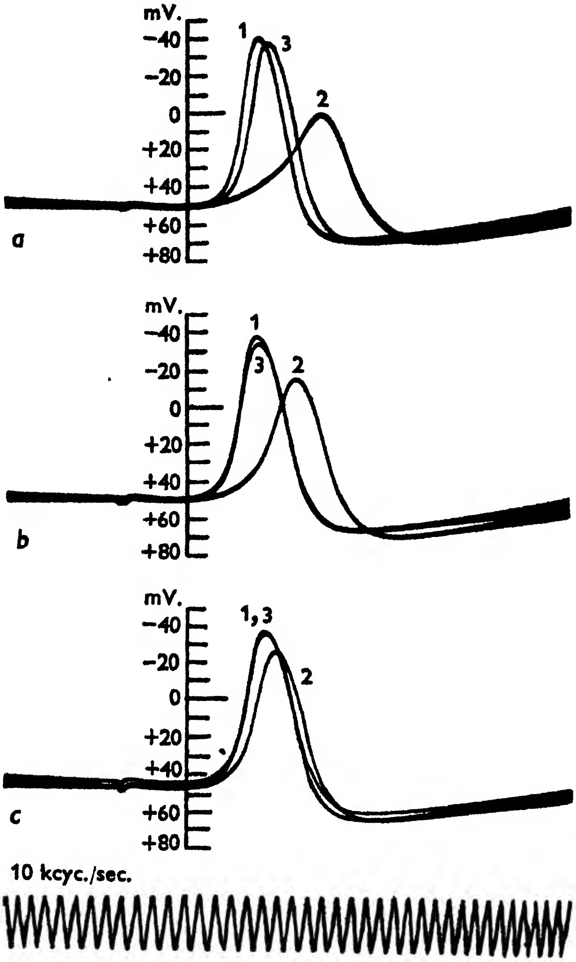


Fig. 4. Action of sodium-deficient solutions on the resting and action potential. *a* 1, response in sea water; *a* 2, after 16 min. in 33 % sea water, 67 % isotonic dextrose; *a* 3, 13 min. after reapplication of sea water. *b* 1, response in sea water, *b* 2, after 15 min. in 50 % sea water, 50 % isotonic dextrose; *b* 3, 6 min. after reapplication of sea water. *c* 1, response in sea water; *c* 2, after 16 min. in 71 % sea water, 29 % isotonic dextrose; *c* 3, 7 min. after reapplication of sea water. The scale gives the potential difference across the nerve membrane (outside − inside) with no allowance for the junction potential between the axoplasm and the sea water in the microelectrode.

recording cell. Records *b* 1, *b* 2, and *b* 3 or *c* 1, *c* 2, and *c* 3 were made in a comparable manner, except that the test solutions consisted of 50 or 71 % sea water. The effects produced by these solutions were smaller, but of the same general type as those illustrated by *a* 1, *a* 2 and *a* 3.

This experiment illustrates a number of important points. In the first place it shows that dilution of sea water with isotonic dextrose caused a large and reversible decrease in the amplitude of the action potential. On the other hand

TABLE 4. Effects of replacing sea water with solutions containing different concentrations of sodium

Concentration of sodium in test solution (expressed as fraction of concentration in sea water)	Change in resting membrane potential (mV.)	Change in action potential (mV.)	Change in active membrane potential (mV.)	Change in positive phase (mV.)	Maximum rate of rise of spike (relative to normal)	Maximum rate of fall of spike (relative to normal)	Action potential amplitude (relative to normal)
0·2	+2	−57	+59	−4	0·08*	0·25*	0·24
	+6						
	(+4)	(−57)	(+59)	(−4)	(0·08*)	(0·25*)	(0·24)
0·33	+2	−42	+44	+1	0·22	0·51	0·54
	(+2)	(−42)	(+44)	(+1)	(0·22)	(0·51)	(0·54)
0·50	0	−19	+19	+2	—	—	0·79
	−1	−23	+22	+2	0·54	0·78	0·73
	+2	−22	+20	+2	0·51	0·77	0·76
	+3	−19	+22	+1	0·51	0·82	0·76
	+4	−19	+23	+1	0·51	0·80	0·75
	+3	−14	+17	+3	0·59	0·80	0·84
	(+2)	(−19)	(+21)	(+2)	(0·53)	(0·79)	(0·77)
0·715	−2	−10	+ 8	+1	0·70	0·83	0·87
	+1	− 8	+ 9	+1	0·74	0·96	0·90
	—	− 9	—	+1	0·79	0·95	0·89
	(0)	(− 9)	(+ 9)	(+1)	(0·75)	(0·91)	(0·89)
1·26	+1	+ 4	− 3	0	1·17	1·11	1·05
	(+1)	(+ 4)	(− 3)	(0)	(1·17)	(1·11)	(1·05)
1·56	0	+ 6	− 6	+1	—	—	1·07
	−1	+ 9	−11	0	—	—	1·12
	−1	+ 8	− 9	+3	—	—	1·10
	−3	+ 7	−10	+2	1·19	1·09	1·09
	—	—	—	—	1·39	1·27	—
	—	—	—	—	1·33	1·19	—
	—	—	—	—	1·27	1·09	—
	(−1)	(+ 7)	(− 9)	(+1)	(1·30)	(1·16)	(1·09)

All rates except those marked with an asterisk were obtained by electrical differentiation. For the purpose of this table the action potential and positive phase are both regarded as positive quantities. A positive change in membrane potential means that the outside of the nerve becomes more positive with respect to the inside. Sodium-deficient solutions were made by diluting sea water with isotonic dextrose, sodium-rich solutions by adding solid sodium chloride to sea water. Average values are enclosed in parentheses. Changes were measured with reference to a normal value in sea water which was obtained in each case from the mean of determinations made before and after application of a test solution.

the resting potential was altered to such a small extent that no difference can be seen in Fig. 4. There was usually a small increase in resting potential, as may be seen from the figures in Table 4, but the change was always small compared to the change in spike amplitude. The constancy of the resting potential means that removal of external sodium reduces the action potential

by decreasing the overshoot or membrane reversal. In fact, in 33% sea water, the overshoot had disappeared and the action potential was then slightly smaller than the resting potential. Another interesting point is that the rate of rise of the action potential was markedly affected by sodium-deficient solutions, whereas the rate of fall changed only in proportion to the amplitude. It can also be seen that the positive phase was only slightly affected by removal of sodium.

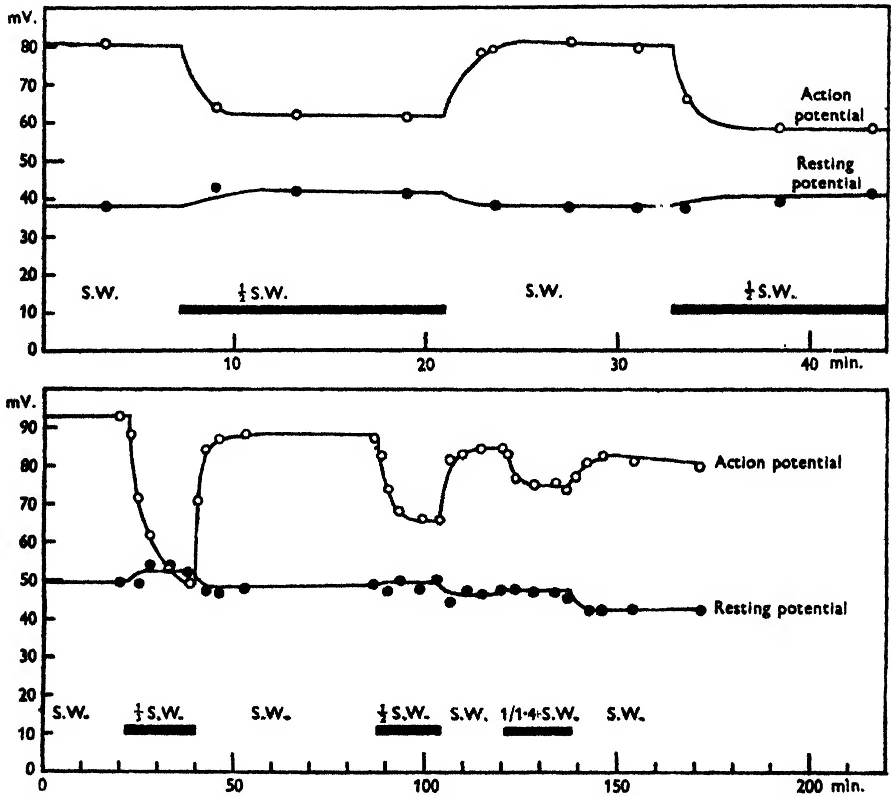


Fig. 5. Time course of action of sodium-deficient solutions made by diluting sea water with isotonic dextrose. Resting potentials are apparent potentials corrected for liquid junction potentials in the external circuit, but not corrected for the junction potential between axoplasm and sea water at the tip of the microelectrode. Both action potential and resting potential are treated as positive quantities.

The quantitative results obtained with sodium-deficient solutions are shown in Table 4. The principal difficulty in making these measurements was connected with the fact that diffusion times prevented the sodium-deficient solutions from acting instantaneously, and it was essential that quantitative measurements should not be made until equilibrium had been obtained. Photographic records

were usually made at intervals of 2, 5, 10 and 15 min. after application of the new solution. This procedure gave satisfactory results when solutions containing more than 50% sea water were employed. The results at 10 and 15 min. rarely differed by more than 2 mV., and equilibrium was sometimes attained after 5 min. On the other hand, measurements in solutions containing less than 50% sodium were unsatisfactory, because there was always a progressive decline in the action potential and in the resting potential. This could not be attributed to diffusion in the space outside the axon, but may have been caused by a slow leakage of potassium chloride from the axon itself. The errors introduced by this progressive decline are not likely to be large, but it is certain that measurements of potentials in 30 and 20% sea water cannot be regarded with the same confidence as can those in 50 and 70% sea water.

The time course of the action of sodium-deficient solutions on both action potential and resting potential is shown by two experiments illustrated in Fig. 5. The resting potential may be seen to undergo small and irregular variations and in general these were accentuated by the operation of changing the solution in the recording cell. Such variations are regarded as spurious, and an attempt has been made to minimize their effect by using average values and neglecting measurements made shortly after the solutions had been changed.

The effect of sodium-deficient solutions on spike height is illustrated by Fig. 6, and the average effect on membrane reversal by Fig. 7. The dotted line in these figures shows the relation which would be obtained if the active membrane behaved like a sodium electrode. In this case the potential difference across the active membrane should be given by

$$E=\frac{RT}{F}\log_e\frac{(\mathrm{Na})_{\mathrm{inside}}}{(\mathrm{Na})_{\mathrm{outside}}}=58\ \mathrm{mV.}\times\log_{10}\frac{(\mathrm{Na})_{\mathrm{inside}}}{(\mathrm{Na})_{\mathrm{outside}}},\qquad(1)$$

where E is the potential of the external solution minus that of axoplasm; R, T and F have their usual significance; $(\mathrm{Na})_{\mathrm{inside}}$ and $(\mathrm{Na})_{\mathrm{outside}}$ are sodium concentrations—or more strictly sodium activities—in the axoplasm and external solution. The change in active membrane potential which results from an alteration of external sodium should be given by equation 2, since it may be assumed that the internal concentration of sodium does not change, or changes only very slowly when the external sodium is altered.

$$\Delta E=E_{\mathrm{test}}-E_{\mathrm{sea\ water}}=\frac{RT}{F}\log_e\frac{(\mathrm{Na})_{\mathrm{sea\ water}}}{(\mathrm{Na})_{\mathrm{test}}}.\qquad(2)$$

The absolute magnitude of the action potential is equal to the difference between the membrane potentials of resting and active nerve. Since the resting potential is only slightly altered by dilution of sea water, equation 2

should also apply to the change in spike height. (The sign of the change must be reversed if the spike is regarded as a positive quantity.) The data in Table 4, Figs. 6 and 7 show that equation 2 is obeyed reasonably by solutions containing 50 and 70 % of the normal sodium concentration. The rough agreement must not be pressed, because the behaviour of the active membrane is likely to be much more complicated than that of a sodium electrode. Another reason for caution is that there is no certain information about the activity coefficient of

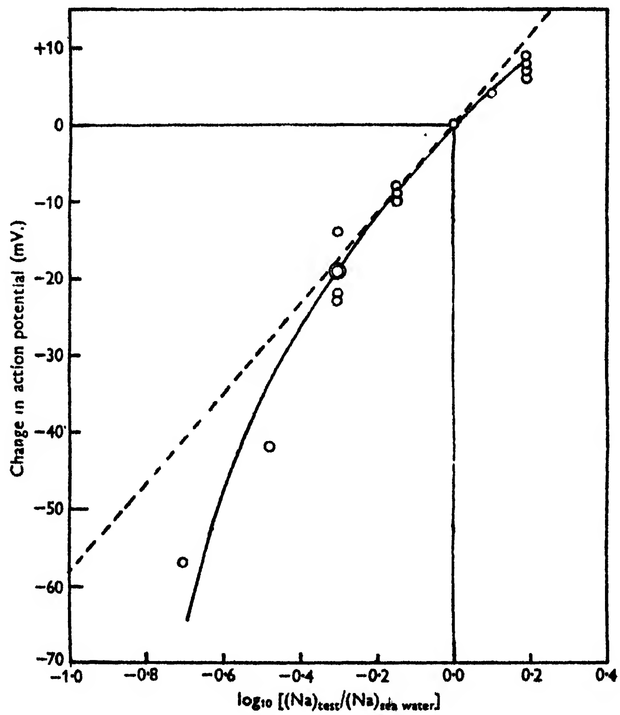


Fig. 6. Change in amplitude of action potential (ordinate) caused by alteration of external sodium concentration (abscissa). The dotted line is drawn through the origin with a slope of 58 mV.

the sodium ion in dextrose mixtures. Preliminary measurements indicate that an allowance for changes in activity coefficients would make the slope of the theoretical line about 10 % less than that in Figs. 6 and 7. But the corrections for activity coefficients were so uncertain that we have preferred to use concentrations in equation 2. The results in Figs. 6 and 7 indicate that the depression of action potential height was disproportionately large in solutions containing 20 and 33 % sea water. An effect of this kind can be explained if it is assumed that the permeability to sodium increases with the depolarization of the membrane. The action potentials in solutions of 20 or 33 % sodium were

much smaller than normal, so that it is plausible to suppose that the mechanism responsible for transporting sodium might not be operating at full efficiency in these solutions.

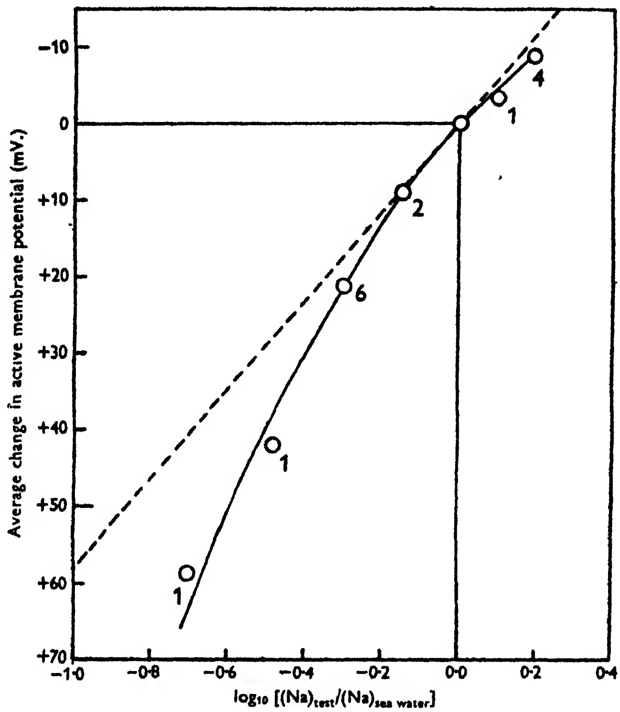


Fig. 7. Average change in active membrane potential (ordinate) caused by alteration of external sodium concentration (abscissa). The dotted line is drawn according to equation 2. The figures attached to the circles show the number of experiments on which each point is based.

Sodium-rich solutions

The experiments with sodium-deficient solutions were in good agreement with the simple hypothesis which they were designed to test. But it might be argued that the results observed were due to the abnormal nature of the external media rather than to any specific effect of the sodium ion. The regular and reversible nature of the changes speaks against this view, but there is a more compelling reason for rejecting it. The concentration of sodium chloride was increased from 455 to 711 mM. by dissolving 15 g. of solid NaCl in 1 l. of sea water. This solution was strongly hypertonic and damaged the axon by osmotic effects in 5–15 min. But before the osmotic effects became apparent the axon gave an increased action potential with characteristics which were the converse of those in sodium-deficient solutions. The effect of sodium-rich solutions is best illustrated by the behaviour of an axon from which almost all the external tissue had been removed by dissection. In this axon the thickness

of the external tissue was about 25 μ. so that diffusion times were relatively short. Fig. 8*a* shows the action potential observed when this axon was immersed in sea water. A sodium-rich solution was applied 2 min. after curve *a* had been obtained, and curve *b* recorded 50 sec. later. The height of the action potential, the overshoot and the rate of rise all show small but quite definite increases. Measurements indicate that the action potential increased from 86 to 95 mV., while the active membrane potential changed from -42 to -53 mV. These values were maintained for 4 min. and, on replacing sea water, returned to

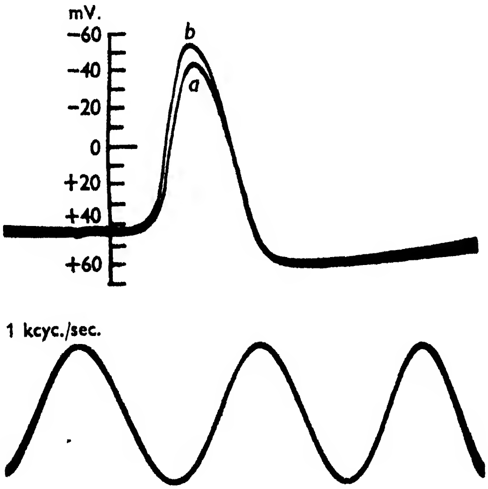


Fig. 8. Action of sodium-rich solution on the resting and action potential. *a*, response in sea water; *b*, response 50 sec. after application of sea water containing additional quantity of NaCl. (The sodium concentration of this solution was 1·56 times that of sea water.) The scale gives the potential difference across the nerve membrane (outside − inside) with no allowance for the junction potential between the axoplasm and the sea water in the micro-electrode.

84 and -41 mV. The changes are not large, but the increase in overshoot is close to that predicted by equation 2. The sodium concentration of this solution was 1·56 times that in sea water so that the theoretical change in overshoot is

$$\Delta E = E_{\text{test}} - E_{\text{sea water}} = 58 \text{ mV.} \times \log_{10} 1/1{\cdot}56 = -11 \text{ mV.}$$

A control with a solution containing 0·5 mol. dextrose dissolved in 1 l. of sea water gave no immediate increase in spike height but only a very small and gradual decrease which must be regarded as an osmotic effect.

Other experiments with sodium-rich solutions gave results which were essentially similar to those in Fig. 8, although the changes observed were somewhat smaller, as may be seen in Table 4. It was also found that the period of increased spike height was rarely maintained for more than a few minutes, and was followed by a period of progressive deterioration which was

only partially reversible. Control experiments with solutions containing extra dextrose showed the phase of progressive deterioration, but never gave the initial increase in spike height, or rate of rise. There is therefore some reason for believing that the changes produced by excess of sodium would have been rather larger if the action of extra sodium could have been dissociated from the osmotic effect of the solutions.

The rate of rise of the action potential

The basic assumption in our hypothesis is that excitation causes the membrane to change from a condition in which the permeability to potassium is greater than the permeability to sodium, to a state in which the permeability to sodium exceeds that to potassium. The transition from the resting to the active state occurs as the resting nerve becomes depolarized by local circuits spreading from an adjacent region of active nerve. Because the inside of the axon contains a low concentration of sodium, external sodium ions should enter the axon at a relatively high rate when excitation occurs. In the absence of other processes, sodium entry would continue until the inside of the axon became sufficiently positive to overcome the effect of the diffusion gradient. The rate at which the membrane approaches its new equilibrium value should be determined by the rate at which the membrane capacity is discharged by entry of sodium. Our hypothesis therefore suggests that the rate of rise of the action potential should be determined by the rate of entry of sodium, and on a simple view it might be expected to be roughly proportional to the external concentration of sodium.

A quantitative basis for part of this argument can be provided in the following way. The membrane current during the action potential is proportional to the second derivative of potential with respect to time, and is therefore zero when the first derivative is at a maximum or a minimum. The current passing through the membrane consists of capacity current ($C\,\partial V/\partial t$), which involves a change of ion density at its outer and inner surface, and an ionic current due to transport of ions across the membrane. These two components must be equal and opposite when the total membrane current is zero. The following relation therefore applies at the moments when the rate of change of membrane voltage is at a maximum or minimum

$$-C\,\partial V/\partial t = I, \tag{3}$$

where I is the net inward current per sq.cm. due to transfer of ions from outside to inside, C is the membrane capacity per sq.cm., V is the potential difference across the nerve membrane (outside potential − inside potential).

The simple nature of equation (3) indicates that the most valuable type of rate measurement is a determination of the maximum rate. This can be obtained by graphical analysis of the action potential, but is best recorded

directly by electrical differentiation. Typical records obtained by this procedure are given in Figs. 9 and 10. These show two distinct phases and not three, as might have been expected from the diphasic character of the squid action potential. The reason for this is that the rate at which the positive phase disappears is small compared to the rates at which the initial part of the spike

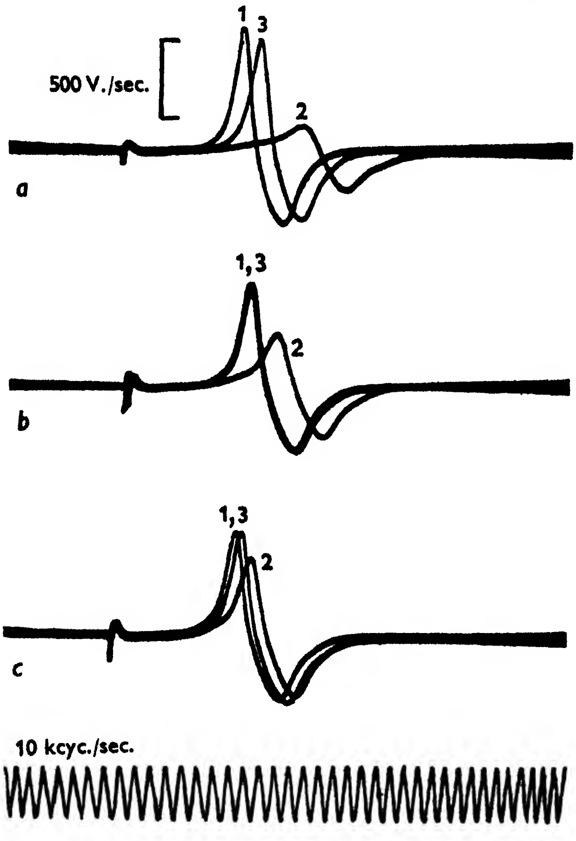


Fig. 9. Action of sodium-deficient solutions on rate of change of membrane voltage. *a* 2, *b* 2, *c* 2, response in 33, 50 and 71 % sea water, remainder in pure sea water. The times at which records were obtained are approximately the same as those in Fig. 4. The calibration scale in *a* applies to all records.

rises and falls. The peak of the rate record is proportional to the positive ionic current entering the axon and the trough to the positive ionic current leaving the axon. The absolute value of these currents can be estimated roughly, since the membrane capacity of the squid axon has been determined as 1·1 μF.cm.$^{-2}$ (Cole & Curtis, 1938) or 1·8 μF.cm.$^{-2}$ (Cole & Curtis, 1939), and may be taken as 1·5 μF.cm.$^{-2}$. The average values for the maximum rates of rise and fall of the spike were 630 and 380 V.sec.$^{-1}$, so that the ionic current entering the axon during the rising phase was of the order of 0·9 mA.cm.$^{-2}$, whereas the ionic

current leaving during the falling phase was of the order of 0·6 mA.cm.$^{-2}$. Corresponding figures in terms of the rate of entry or exit of a monovalent kation are 10^{-8} and $0{\cdot}6 \times 10^{-8}$ mol.cm.$^{-2}$ sec.$^{-1}$.

Fig. 9 shows how the first derivative of the action potential is affected by sodium-deficient solutions. These records were obtained from the same axon and under the same conditions as those in Fig. 4; they show that the rate of rise of the action potential undergoes large and substantially reversible changes as a result of treatment with sodium-deficient solutions. Fig. 10 shows the changes produced by successive application of solutions containing 50, 100 and 156 % of the normal sodium concentration. The action potential reached

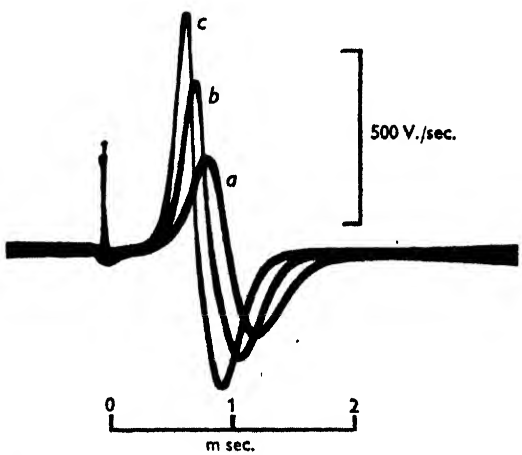


Fig. 10. Rate of change of membrane voltage in solutions containing various concentrations of sodium: *a*, 12 min. after application of 50 % sea water, 50 % isotonic dextrose; *b*, 16 min. after application of sea water; *c*, 2 min. after application of sodium-rich solution (the sodium concentration in this solution was 1·56 times that in sea water). The interval between record *a* and *b* was 18 min. and between *b* and *c* was 3 min.

a constant value in the 50 and 100 % solutions so that the change in rate shown by record *c* was certainly a genuine increase and not merely a recovery from the previous immersion in the 50 % solution. Data from other axons are collected in Table 4 and plotted graphically in Fig. 11. It will be seen that the rate of rise is proportional to sodium concentration over the range 50–100 % sea water, but that the rate falls off rapidly below 50 %. This effect is almost certainly related to the disproportionately large changes in action potential observed in solutions containing 20 and 33 % sea water.

The rates of rise showed substantial increases in solutions containing extra sodium, but the effects were no longer proportional to the sodium concentration. Thus the largest increase encountered in a solution containing 1·56 times the normal sodium was 1·39, and the average value was only 1·3. This result may be attributed to the deleterious action of the hypertonic solutions, but it is also

possible that there may be a genuine lack of proportionality in solutions containing an excess of sodium. However, there is good evidence to show that simple proportionality does hold over a limited region and it is certain that the rate of rise is altered reversibly over a wide range by changes in the external sodium concentration.

The rate of fall of the action potential is also influenced by sodium, but to a lesser extent (Table 4). Thus the average change in rate of rise in a 50% solution was 0·53, whereas the average change in rate of fall was 0·8. The rates of fall appear to change in proportion to the height of the action potential, as

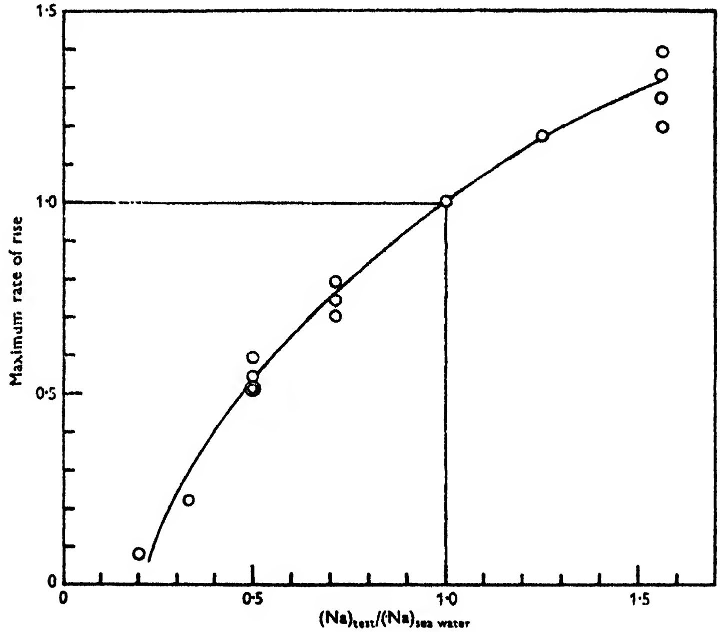


Fig. 11. Ordinate: maximum rate of rise of spike in test solution/maximum rate of rise in sea water. Abscissa: sodium concentration of test solution/sodium concentration in sea water.

may be seen by comparing the average ratios in the last two columns of Table 4. This result suggests that changes in external sodium affect the rate of fall indirectly by altering the amplitude of the spike. A change in the rate of fall is a natural consequence of a change in spike height; for it is to be expected that the rate at which the potential reverts to its resting value should depend upon the extent to which activity has displaced the membrane from its resting level.

Changes in conduction velocity

The velocity of transmission should be reduced by sodium-deficient solutions, since these solutions lower the rate of rise of the action potential. This deduction could not be tested in the experiments with internal electrodes, since a large

part of the conduction time arose in the lower part of the nerve which was immersed in oil. A single experiment with external electrodes was made in order to find out if there was an appreciable change of velocity in a large volume of 50% sea water. The axon was arranged in such a way that the conduction stretch between stimulating and recording electrodes could be dipped into sea water or into a test solution (cf. Hodgkin, 1939). With an arrangement of this kind the absolute changes in conduction time could be measured with considerable accuracy, but there was some uncertainty in determining the velocity because it was difficult to know which was the correct point on the spike to choose for measurement. This source of error can be eliminated by the method used by Katz (1947), but shortage of time and material prevented us from making more than a single experiment of the simplest kind. The result left no doubt that there was a substantial decrease of conduction velocity in a solution containing 50% sea water, and a tentative estimate is that the velocity in this solution was 0·7 of that in sea water. The change in velocity was evidently smaller than the average change in rate given in Table 4, but there is no reason to suppose that the velocity should change as the first power of the rate of rise, and in a simplified theoretical system it can be shown to vary with the square root of the rate of rise of the action potential. There is evidently a difference between this result and those recently reported by Katz (1947) for *Carcinus* axons. Katz was primarily concerned with another aspect of the problem of conduction velocity, but two experiments are quoted which show a velocity decrease of only 5% in a solution containing 50% sea water and 50% isotonic sucrose, and in other unpublished experiments of a similar kind the velocity change rarely exceeded 10%. The data at present available are not sufficient to justify speculation into the nature of this apparent difference between crab and squid fibres. But the conduction velocity must depend upon processes occurring at threshold as well as upon the rate of rise or height of the action potential, and it is likely that dilution of sea water would give different overall effects in different types of axon. In this connexion it should be remembered that dilution of sea water with sugar solutions alters the concentration of other ions besides that of sodium, and it is conceivable that the effect of sodium removal may sometimes be balanced by an increase in excitability resulting from the simultaneous reduction of calcium concentration. Apart from the numerical discrepancy, the results of Katz are in general agreement with those reported here. Thus the velocity of conduction in *Carcinus* axons was found to undergo a substantial decrease in solutions containing less than 30% sea water and block occurred when the sea-water content was less than 10%.

Specificity of sodium action

The reduction in spike height which results from mixing sea water with isotonic dextrose has been attributed to dilution of sodium, but it is conceivable that the observed effects might have been partly due to dilution of other ions such as Ca, K or Cl. This possibility was examined by comparing the effects of two solutions. The first solution was made by mixing artificial sea water with isotonic dextrose, while the second was made in such a way that all components except the sodium and chloride ions were maintained at about their normal level. The composition of the two solutions and the results obtained with them are given in Table 5. It will be seen that solution 2 gave a smaller action potential than solution 1, but that this drop was almost entirely due to a 5 mV. diminution in resting potential which probably arose from the increased potassium concentration of the second solution. Since both solutions contained the same concentration of sodium, equation (2) predicts that the active membrane potential should remain constant and the figures in Table 5 show that this prediction is verified. The rate of rise in the second solution was 20 % less than that in the first, and this effect may be attributed either to the higher calcium and magnesium content of solution 2 or to the lowered resting potential resulting from the increase in potassium concentration.

TABLE 5.

Operation	Change in resting membrane potential (mV.)	Change in action potential (mV.)	Change in active membrane potential (mV.)	Change in positive phase (mV.)	Maximum rate of rise (relative to previous condition)	Maximum rate of fall (relative to previous condition)	Action potential amplitude (relative to previous condition)
From artificial sea water to solution 1	+4	−19	+23	+1	0·51	0·80	0·75]
From solution 1 to solution 2	−5	− 5	0	0	0·81	0·92	0·90]
From artificial sea water to solution 1	+3	−14	+17	+3	0·59	0·80	0·84 }
From solution 1 to solution 3	+1	+ 2	− 1	0	1·17	1·09	1·03 }

Square brackets indicate that measurements were made on the same axon but were separated by a considerable time interval; curved brackets that they were obtained on the same axon at approximately the same time. All figures are average values determined in the usual way. The compositions of the solutions are given below:

Solution	Description	Concentration as fraction of concentration in artificial sea water: Na	K	Ca	Mg	Cl	HCO_3
1	Artificial sea water diluted 1:1 with isotonic dextrose	0·5	0·5	0·5	0·5	0·5	0·5
2	Solution 1 + K, Ca, Mg, HCO_3	0·5	1·0	1·0	1·0	0·5	1·0
3	Artificial sea water diluted 1:1 with 0·6 M-choline chloride	0·5	0·5	0·5	0·5	1·0	0·5

The previous experiment indicates that the changes in active membrane potential and spike height were primarily due to alterations in the concentrations of either the chloride or the sodium ion. The effect of these two ions may be separated by diluting sea water with isotonic choline chloride instead of isotonic dextrose. The results obtained in a single experiment of this type are illustrated by the effects given in Table 5 for solutions 1 and 3. It will be seen that the general action of these solutions was similar and, in particular, that the active membrane potentials differed by less than 1 mV., although the chloride concentrations of the two solutions were widely different. The only anomalous point is that the rate of rise was found to be appreciably greater in the solution containing choline chloride than in the one containing dextrose. Part of this difference may be attributed to a small change in resting potential, but it seems unreasonable to ascribe all the increase to this cause.

A single experiment with an artificial sea-water solution containing lithium instead of sodium indicated that the action of these two ions was almost identical. This result is supported by unpublished experiments with *Carcinus* axons which show that propagation occurs satisfactorily for at least 1 hr. in a solution containing lithium but no sodium. Gallego & Lorente de Nó (1947) report that medullated nerve becomes depolarized and inexcitable after immersion in lithium solutions for several hours. We must therefore suppose either that the reactions of vertebrate nerve to lithium differ from those of invertebrate nerve, or that our experiments were not maintained for sufficient time to reveal the effects described by Gallego & Lorente de Nó.

Preliminary experiments with isotonic sucrose mixtures show that the action of this sugar was similar to that of dextrose.

No perceptible changes occurred when the oxygen tension of the sea water was increased fivefold.

Effect of varying potassium concentration

The action potential may be regarded as being made up of a component due to the resting potential, which is only slightly altered by dilution of sea water with isotonic dextrose, and an overshoot which is directly influenced by the external sodium concentration. It is known that variations in the external potassium concentration alter the resting potential, and on a simple view it is to be expected that these variations would change the amplitude of the action potential but not the reversed potential difference of the active membrane. This hypothesis cannot be pressed, because increasing the potassium concentration causes nerve fibres to become inexcitable long before they are completely depolarized (Curtis & Cole, 1942). There is also the experimental difficulty that the changes in resting potential are small over the range in which excitability is retained. In practice, this meant that the values of resting potential and spike height had to be measured to a degree of precision which was near the experimental limit.

The effect of changing from a potassium-free solution to one containing the normal potassium content is shown in Fig. 12. It will be seen that the action potential was slightly greater in the potassium-free solution than it was in sea water, and that this effect was largely due to a change in the resting potential. The numerical results obtained in this and other experiments are given in Table 6 and are more reliable than values obtained by comparison of single records of the type shown in Fig. 12. They show that the action potential in a potassium-free solution was 4·5 mV. greater than normal and that the resting

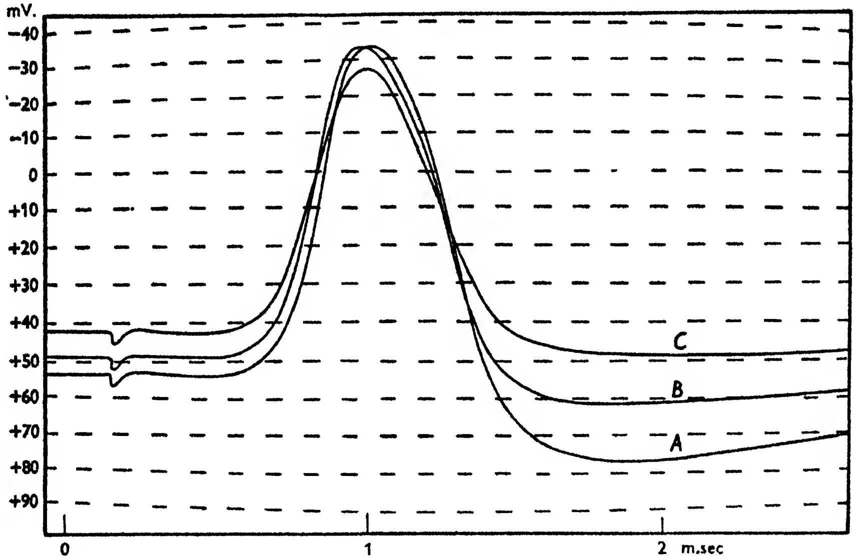


Fig. 12. Effect of varying K concentration on action potential and resting potential. Curve *A*, K-free artificial sea water (K = 0, Na = 463 mM.); *B*, artificial sea water (K = 10 mM., Na = 453 mM.); *C*, K-rich artificial sea water (K = 20 mM., Na = 443 mM.). The dotted lines were traced from a projection of the calibration grid and give the potential difference across the nerve membrane (outside − inside) without correction for the junction potential between the axoplasm and the sea water in the microelectrode.

potential was 3·1 mV. larger. The active membrane was therefore 1·4 mV. more negative in a potassium-free solution than in sea water. The sodium concentration of the first solution was 2·2 % greater than that of the second, so that 0·5 mV. of the difference must be attributed to this cause. The remaining difference is not greater than the experimental error, but is probably real because the converse effect is seen with potassium-rich solutions. Thus, in a solution containing twice the usual amount of potassium, the decrease in resting potential accounts for less than half the decrease in action potential, and further increases in potassium concentration cause the spike to drop rapidly to zero, although there is still a substantial resting potential. These facts indicate that it is an over-simplification to suppose that the active

membrane always reaches the equilibrium potential of a sodium electrode. Instead, we must assume that the sodium permeability does not rise to a value high enough to swamp the contribution of potassium and chloride to the active membrane potential. Under these circumstances anything which interferes with the sodium transport mechanism must result in a diminution of spike height. If this view is adopted the changes in Table 6 can be explained by supposing that the efficiency of the sodium transport mechanism depends upon the membrane potential of the resting nerve. Raising the resting membrane potential may cause a slight increase in efficiency, whereas decreasing it leads to a rapidly augmenting drop in efficiency. A hypothesis of this kind is also needed to explain the fact that strong cathodic currents depress the peak of the action potential more than they decrease the resting potential of *Carcinus* axons. This phenomenon is conspicuous when a train of spikes is recorded from the stimulating electrode and is illustrated by Fig. 1 of a recent paper (Hodgkin, 1948).

Small changes in potassium concentration have a marked effect on the positive phase of the squid potential. In Fig. 12 the positive phase in K-free solution amounted to 23 mV. whereas in 2 K it was only 7 mV. Table 6 shows that this action of potassium is consistent and repeatable. A theoretical explanation of the effect is given on p. 70.

TABLE 6. Effect of solutions of varying potassium content

Characteristics in artificial sea water [Na = 453 mM., K = 10 mM.] (in mV.)

	Resting potential (r.p.)	Action potential (a.p.)	Active membrane potential (a.m.p.)	Positive phase (p.p.)
	+46	+86	−40	+12
	+41	+83	−42	+15
	+48	+82	−34	+14
	+48	+83	−35	+12
Average	+46	+84	−38	+13

Change in potential on substituting test solution for artificial sea water (in mV.)

	K-free [Na = 463 mM., K = 0.]				1½K [Na = 448 mM., K = 15 mM.]				2K [Na = 443 mM., K = 20 mM.]			
	Δr.p.	Δa.p.	Δa.m.p.	Δp.p.	Δr.p.	Δa.p.	Δa.m.p.	Δp.p.	Δr.p.	Δa.p.	Δa.m.p.	Δp.p.
	+2·5	+4·2	−1·7	+10·0	−1·7	−2·7	+1·0	−3·2	−3·6	−6·5	+2·9	−6·2
	+1·7	+4·5	−2·8	+10·9					−2·1	−7·3	+5·2	−5·7
	+4·2	+4·6	−0·4	+9·6	−3·0	−4·5	+1·5	−4·2				
	+3·9	+4·7	−0·8	+10·3					−5·5	−12·1	+6·6	−5·9
Average	+3·1	+4·5	−1·4	+10·2	−2·3	−3·6	+1·2	−3·7	−3·7	−8·6	+4·9	−5·9

DISCUSSION

The experiments described in this paper are clearly consistent with the view that the active membrane becomes selectively permeable to sodium, and thereby allows a reversed membrane e.m.f. to be established. The evidence is

indirect, and the sodium hypothesis cannot be pressed until more is known about the ionic exchanges associated with nervous transmission. But the hypothesis does provide a good working basis for future experiments, and it gives a satisfactory explanation of several observations which cannot be reconciled with the classical membrane theory. On the other hand, the hypothesis encounters a number of difficulties of which only a few can be mentioned here. One of the most serious objections arises from the fact that Curtis & Cole (1942) describe an experiment in which the active membrane reversed by 110 mV., whereas Steinbach & Spiegelman's (1943) figures indicate that the sodium concentration of fresh axoplasm is about one-tenth of that in sea water. The maximum overshoot allowed by a tenfold ratio is 58 mV. and the ratio would have to be nearly 100 in order to produce an overshoot of 110 mV. The discrepancy is all the more serious because it is exceedingly unlikely that the membrane potential could reach the theoretical maximum for a sodium electrode. The difficulty does not arise in our experiments, since the reversed membrane e.m.f. has always been well below the limit allowed by Steinbach & Spiegelman's figures. The only alternatives which remain if Curtis & Cole's figure of 110 mV. is accepted are: first, that the sodium hypothesis is incorrect or incomplete; and secondly, that the sodium-ion activity in certain axons may be less than one-hundredth of that in the external fluid. Another possible criticism is that many agents affect the amplitude of the action potential without causing much change in the magnitude of the resting potential. Examples are afforded by cocaine or amyl alcohol, which block conduction at concentrations that cause a slight increase in resting potential (Bishop, 1932). Observations of this kind can be explained by assuming that the mechanism for transporting sodium is of a highly specialized nature, and is readily put out of action by agents which have little effect on the resting potential. Another possibility is that certain substances may act on the secretory mechanism which normally keeps the internal sodium at a low level.

For many years physiologists have known that the action potential of medullated nerve is ultimately abolished by anoxia or by agents which interfere with oxidative processes (Gerard, 1932; Schmitt, 1930; Schmitt & Schmitt, 1931; Lorente de Nó, 1947). But agents of this type also reduce the resting potential and, in such cases, the action potential of medullated nerve can be restored by repolarizing the nerve with an anodic current (Lorente de Nó, 1947). There is therefore little reason to believe that the processes directly concerned with the generation of the action potential are of an oxidative nature. The converse view is expressed by Arvanitaki & Chalazonitis (1947) as a result of an interesting investigation into the effect of metabolic inhibitors on *Sepia* nerve. But the axons used in these experiments were surrounded by a relatively small amount of external fluid and stimulation frequencies of the order of 100 per sec. were employed. Under these conditions secretory activity

may be of great importance for the maintenance of ionic concentration differences, and hence for the maintenance of normal excitability. There is, in any case, no direct conflict between the views of Arvanitaki & Chalazonitis and our own, since it is conceivable that oxidative metabolism may be essential for the proper operation of the mechanism responsible for transport of sodium.

The last objection to be mentioned is of a different kind. It has been assumed that the resting membrane is permeable to potassium and to chloride, but impermeable or only sparingly permeable to sodium. This is a plausible assumption since sodium is a more heavily hydrated ion than potassium or chloride. On the other hand, it is much more difficult to accept the assumption that the active membrane can become selectively permeable to sodium. We therefore suggest that sodium does not cross the membrane in ionic form, but enters into combination with a lipoid soluble carrier in the membrane which is only free to move when the membrane is depolarized. Potassium ions cannot cross the membrane by this route, because their affinity for the carrier is assumed to be small. An assumption of this kind is speculative but not unreasonable, since there is already some indication that a specific, enzyme-like process is concerned with the transport of sodium through cell membranes (Davson & Reiner, 1942; Krogh, 1946; Ussing, 1947). In this connexion it is interesting to read that the permeability of the erythrocyte of the cat to sodium may be five to ten times greater than the permeability to potassium (Davson & Reiner, 1942), and that sodium permeability is reduced to zero by concentrations of amyl alcohol which cause an increase in potassium permeability (Davson, 1940).

In formulating our hypothesis we have been careful to avoid making any quantitative assumptions about the relative permeabilities of the membrane to sodium and potassium. The resting membrane has been considered as more permeable to potassium than sodium, and this condition was regarded as reversed during activity. It is natural to inquire whether any limit can be set to the degree of selective permeability actually present in the resting and active membranes. Some light can be thrown on this problem if the observed potentials are compared with those predicted by a simple equation. In order to interpret the results in terms of selective permeability we need to know the potential difference which would arise across a membrane separating different concentrations of potassium, chloride and sodium. Thermodynamic equations cannot be applied because the system is not in equilibrium, while the theories of Planck (1890*a*, *b*) and Henderson (1907, 1908) make assumptions which are almost certainly not valid for a thin membrane of high resistance. A simple equation has been derived by Goldman (1943). He assumes that the voltage gradient through the membrane may be regarded as constant and that ions move under the influence of diffusion and the electric field. Goldman also makes the tacit assumption that the concentrations of ions at the edges of the

membrane are directly proportional to those in the aqueous solutions. In the Appendix we show that these assumptions give the following expression for the membrane potential:

$$E=\frac{RT}{F}\log_e\left[\frac{P_K\,(K)_i+P_{Na}\,(Na)_i+P_{Cl}\,(Cl)_o}{P_K\,(K)_o+P_{Na}\,(Na)_o+P_{Cl}\,(Cl)_i}\right], \tag{4}$$

where $(K)_i$, $(Na)_i$ and $(Cl)_i$ are activities inside the axon; $(K)_o$, $(Na)_o$ and $(Cl)_o$ are activities outside the axon; P_K, P_{Na} and P_{Cl} are permeability constants for the individual ions. The relative magnitudes of the permeability constants depend upon the relative mobilities and solubilities of the ions in the membrane. Thus

$$P_K=\frac{RT}{Fa}u_K\,b_K;\quad P_{Na}=\frac{RT}{Fa}u_{Na}\,b_{Na};\quad P_{Cl}=\frac{RT}{Fa}u_{Cl}\,b_{Cl},$$

where a is the thickness of the membrane; u_K, u_{Na} and u_{Cl} are mobilities of the ions in the membrane; b_K, b_{Na} and b_{Cl} are the partition coefficients between the membrane and the aqueous solution. E is the potential difference across the membrane (outside − inside) in the absence of any net ionic current.

There are many reasons for supposing that this equation is no more than a rough approximation, and it clearly cannot give exact results if ions enter into chemical combination with carrier molecules in the membrane or if appreciable quantities of current are transported by ions other than K, Na or Cl. On the other hand, the equation has two important advantages. In the first place it is extremely simple, and in the second it reduces to the thermodynamically correct forms when any one permeability constant is made large compared to the others.

In order to apply this equation we must first adopt values for the internal concentrations of K, Cl and Na, and for this purpose the data of Steinbach (1941) and Steinbach & Spiegelman (1943) will be employed. These writers give values for freshly isolated axons and for those treated with sea water for 2–4 hr. The physiological condition of the axons used in the present work is thought to be intermediate between these two conditions and we therefore propose that the following values should be used:

$(K)_i = 345$ mM. (mean of average values in table 4, in Steinbach & Spiegelman, 1943);

$(Na)_i = 72$ mM. (mean of average values in table 4, in Steinbach & Spiegelman, 1943);

$(Cl)_i = 61$ mM. (mean of tables 1 and 2 in Steinbach, 1941).

The experiments of Steinbach (1941) and Steinbach & Spiegelman (1943) suggest that the squid axon is permeable to chloride, sodium and potassium, but they give little information about the relative permeabilities to these three ions. It is extremely unlikely that the permeability ratios can be determined from electrical measurements with any degree of certainty, since the values

adopted for the internal concentration are subject to considerable error, and equation 4 cannot be regarded as more than a rough approximation. Our object is to show that a large number of observations can be fitted into a coherent picture by the use of appropriate permeability ratios for resting, active and refractory nerve. The experimental data against which equation (4) must be tested are summarized in Table 7, which shows the average change in membrane potential produced by substituting a test solution for sea water or

TABLE 7.

State of nerve	Solution	Composition of test solution: K mM.	Na mM.	Cl mM.	Change in membrane potential on substituting test solution for sea water or artificial sea water: Observed mV.	Calculated mV.	Permeability coefficients used in calculation: P_K	P_{Na}	P_{Cl}
Resting	A	0	465	587	+ 3	+ 5	1	0·04	0·45
	B	15	450	587	− 2	− 2			
	C	20	445	587	− 4	− 4			
	D	7	324	384	0	+ 1			
	E	5	227	270	+ 2	+ 2			
	F	3	152	180	+ 2	+ 2			
	G	2	91	108	+ 4	+ 3			
	H	10	573	658	+ 1	0			
	I	10	711	796	− 2	0			
Active (peak of spike)	A	0	465	587	− 1	− 1	1	20	0·45
	B	15	450	587	+ 1	0			
	C	20	445	587	+ 5	+ 1			
	D	7	324	384	+ 9	+ 8			
	E	5	227	270	+21	+16			
	F	3	152	180	+44	+25			
	G	2	91	108	+59	+38			
	H	10	573	658	− 3	− 5			
	I	10	711	796	− 9	−10			
Refractory (maximum of positive phase)	A	0	465	587	+13	+12	1·8	0	0·45
	B	15	450	587	− 6	− 5			
	C	20	445	587	−10	− 9			
	D	7	324	384	+ 1	+ 1			
	E	5	227	270	+ 4	+ 2			
	F	3	152	180	+ 4	+ 3			
	G	2	91	108	0	+ 3			
	H	10	573	658	+ 1	+ 1			
	I	10	711	796	0	+ 3			
Membrane potential at rest in sea water					+48+J	+59	As above		
Membrane potential at height of activity in sea water					−40+J	−38			
Membrane potential at maximum of positive phase					+62+J	+74			
Action potential in sea water					88	97			
Positive phase in sea water					14	15			

Solutions A, B and C were tested against an artificial sea-water solution containing 10 mM-K 455 mM-Na, 587 mM-Cl. Solutions D–I were tested against sea water containing approximately 10mM-K, 455 mM-Na, 540 mM-Cl. Calculated potentials were obtained from equation 4 using values of $(K)_i = 345$ mM., $(Na)_i = 72$ mM., $(Cl)_i = 61$ mM. J is the liquid junction potential between the axoplasm and the sea water in the microelectrode.

artificial sea water. Solution A is potassium-free artificial sea water, solutions B and C are potassium-rich artificial sea water; D, E, F, G are sea-water solutions diluted with isotonic dextrose while H and I were made by adding solid sodium chloride to sea water. It will be seen that there is reasonable agreement between all the results obtained with resting nerve and those predicted by the theory for $P_K:P_{Na}:P_{Cl}=1:0{\cdot}04:0{\cdot}45$. These coefficients were

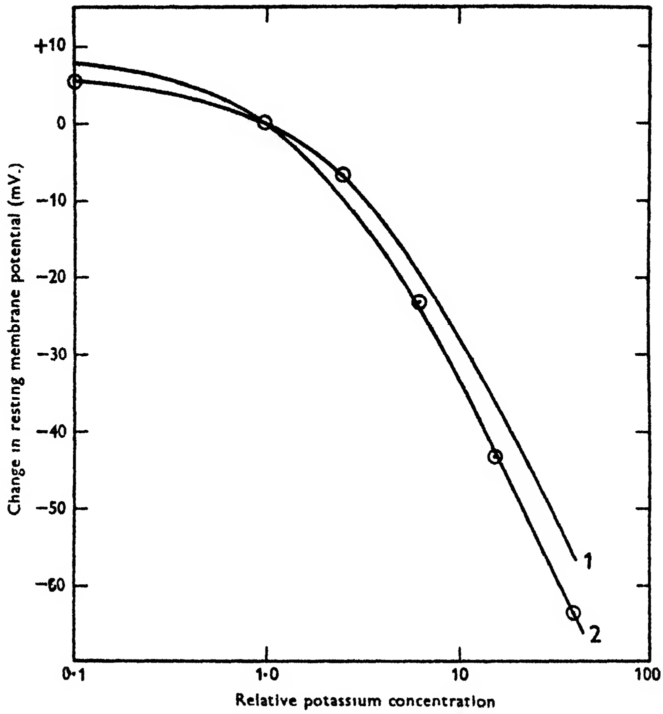


Fig. 13. Data from Curtis & Cole (1942, fig. 2). Ordinate: change in resting membrane potential produced by substitution of test solution for standard sea-water solution containing 13 mM-K. Abscissa: potassium concentration of test solution in multiples of potassium concentration in standard solution (13 mM.); logarithmic scale. Circles were obtained from Curtis & Cole's experimental points. Smooth curves were drawn according to equation 4 with

$P_K:P_{Na}:P_{Cl}=1:0{\cdot}04:0{\cdot}45$ (curve 1) and $P_K:P_{Na}:P_{Cl}=1:0{\cdot}025:0{\cdot}3$ (curve 2).

Internal concentrations assumed to remain constant; $(Cl)_i$ taken as 61 mM. and $(Na)_o$ obtained from the proportion of isotonic sodium chloride present in the test solution.

found by trial and error, and serious deviations occur if values differing by more than 50 % are employed. Thus, if the chloride permeability is made zero, an increase of 17 mV. in the resting potential is predicted for a solution of 50 % sea water, while if it is made equal to the potassium permeability a decrease of 5 mV. is predicted. The average change observed experimentally was +2 mV. and this is the value predicted by the coefficients which have been adopted. The variations in resting potential were not large, because the external

potassium concentration was kept well within the physiological range. However, the relation between potassium concentration and resting potential has been determined by Curtis & Cole (1942) and their data are supported by unpublished results obtained with Mr Huxley in 1939. Curtis & Cole's data are shown by the hollow circles in Fig. 13, while curves 1 and 2 were plotted according to the theory for $P_K : P_{Na} : P_{Cl} = 1 : 0{\cdot}04 : 0{\cdot}45$ and $1 : 0{\cdot}025 : 0{\cdot}3$ respectively. It will be seen that the first curve fits the data obtained in the physiological range, but that rather different values are needed to cover the observations with high potassium concentration. However, the deviations are not large and are hardly surprising in view of the simplifications made in deriving equation 4. The absolute value of the resting potential predicted on the basis of $P_K : P_{Na} : P_{Cl} = 1 : 0{\cdot}04 : 0{\cdot}45$ is 59 mV., while the resting potential observed with a microelectrode containing sea water averaged 48 mV. The difference is most easily explained by supposing that there is a liquid junction potential of 11 mV. between sea water and axoplasm. The magnitude of the liquid junction potential has not been measured experimentally and cannot be computed theoretically until more is known about the nature of the organic anions in the axoplasm. A tentative estimate can be obtained by making the assumption of Curtis & Cole (1942) that the anions are monovalent and have a mobility sufficient to give the axoplasm its measured value of 28 Ω.cm. In this way Curtis & Cole obtained a value of 6 mV. for the junction potential between isotonic KCl and axoplasm. A repetition of this calculation, using the figures for internal potassium, chloride and sodium adopted in this paper, gave a value of 14 mV. for the junction potential between sea water and axoplasm.

The experiments described in this paper indicate that the action potential arises because the sodium permeability increases as the nerve membrane is depolarized. The absolute magnitude of the action potential can be calculated if values are assumed for the relative permeabilities of the active membrane to sodium, potassium and chloride ions. If the permeabilities are assumed to change from a resting condition in which $P_K : P_{Na} : P_{Cl} = 1 : 0{\cdot}04 : 0{\cdot}45$ to an active condition in which $P_K : P_{Na} : P_{Cl} = 1 : 20 : 0{\cdot}45$ an action potential of 97 mV. is obtained. This is 9 mV. larger than the average value observed experimentally, but it must be remembered that equation 4 only applies if there is no external current through the membrane. The difference of 9 mV. appears to be a safe allowance for the potential difference arising from current flow, since the membrane current density at the height of activity is about 0·2 mA.cm.$^{-2}$ (Hodgkin & Huxley, 1945), and the active membrane resistance is about 25 Ω.cm.^{2} (Cole & Curtis, 1939). The new values of the permeability coefficients may be used to predict the changes in potential which would arise from the applications of solutions A–I and these are compared with the average experimental results in Table 7. It will be seen that there is reasonable agreement over most of the range, but that deviations occur in the case of

solution C (twice normal potassium), E (2/3 isotonic dextrose, 1/3 sea water) and F (4/5 isotonic dextrose, 1/5 sea water). The nature of these deviations has already been discussed and requires little further comment. In order to account for them we must assume that, for one reason or another, the active membrane does not acquire its full sodium permeability. Thus a change of 58 mV. would have been predicted for solution G if we had assumed that in this solution the permeability coefficients were 1:2·5:0·45 instead of 1:20:0·45 as in the normal active membrane. Although the sodium permeability has been assumed to be twenty times the potassium permeability, the active membrane potential is still 8 mV. below the theoretical maximum for a sodium electrode. This indicates that equation 1 is useful only in so far as it gives an upper limit to the reversed membrane potential. On the other hand, equation 2 remains a reasonably good approximation since it predicts changes which are within 10% of the calculated values in Table 7.

The third block of figures in Table 7 give the changes in membrane potential recorded at the height of the positive phase. In this condition the nerve is in a refractory state, so that there is no reason to assume that the permeability ratios are intermediate between those in the resting and active states. If the sodium permeability remained at its active level of 20, the nerve could show no recovery from the crest of the action potential. The sodium permeability must therefore be reduced by exhaustion or inactivation of the special mechanism which comes into play when the nerve is first depolarized. We now assume that the sodium permeability is reduced to zero and that it does not recover its normal value until the end of the relative refractory period. If this assumption is made, we find that the nerve would show a positive phase of 10 mV. This is not far from that recorded experimentally, but there are still considerable deviations between theory and experiment which can be resolved by making $P_{K}:P_{Na}:P_{Cl}=1\cdot8:0:0\cdot45$. These values have been adopted in Table 7 and give good agreement both with respect to the absolute magnitude of the positive phase and to its variation in solutions of different potassium content. The agreement may be fortuitous and can hardly be used as evidence for a differential action on potassium and chloride permeability. On the other hand, the assumption that the sodium permeability is reduced to a subnormal value during the recovery process appears to be in keeping with the general nature of the refractory period, and provides a plausible explanation of the characteristic diphasic appearance of the squid action potential. The positive phase is not seen in other single fibre preparations, but it must be remembered that the assumptions which have been made only lead to a positive phase when there is an appreciable leakage of sodium in the resting condition. A fibre with low-sodium leakage and with potassium and chloride ions distributed according to a Donnan ratio would have a membrane potential close to the theoretical maximum for a potassium electrode, which would be relatively insensitive to

a decrease in the amount of sodium leakage. In such fibres the action potential would return to the resting level without showing any appreciable positive phase.

The preceding arguments suggest that an isolated squid axon is not in a steady state, but is gaining sodium and leaking potassium at a rate determined by the permeability of the membrane and the concentration differences across it. An exchange of this kind has been observed by Steinbach & Spiegelman (1943), and it is interesting to compare their result with that predicted by the constant field theory on which equation 4 is based. Steinbach & Spiegelman's figures show an average increase of 50 mM-Na and an average decrease of 72 mM-K during a period of 3 hr. These figures may be expressed in terms of the flow of ions through 1 sq.cm. of membrane, since the average axon diameter cannot have been far from 500 μ. Adopting this value for the diameter we find that the entry of sodium through 1 sq.cm. was 6×10^{-11} mol.sec.$^{-1}$ while the exit of potassium was 8×10^{-11} mol.sec.$^{-1}$. In order to calculate a theoretical flow from the constant field theory we need to know the concentration differences across the membrane, the permeability ratios and the absolute value of the membrane conductance. The relation between these quantities is given by equation 7·0, 7·1 or 7·2 of the Appendix and numerical values can be obtained by adopting the concentrations and permeability coefficients used previously, with a value of 1000 Ω.cm.^{2} for the membrane resistance (Cole & Hodgkin, 1939). The following theoretical rates are obtained: entry Na, $8{\cdot}4 \times 10^{-11}$ mol.cm.$^{-2}$sec.$^{-1}$; exit K, $10{\cdot}6 \times 10^{-11}$ mol.cm.$^{-2}$sec.$^{-1}$; exit Cl, $2{\cdot}2 \times 10^{-11}$ mol.cm.$^{-2}$sec.$^{-1}$. Steinbach & Spiegelman (1943) give no figures for the flow of chloride, but Steinbach (1941) states that the chloride concentration of squid axoplasm shows a rise from an initial value of 36 mM. to one of about 75 mM. at which level the concentration remains constant for long periods of time. If a value of 36 mM. had been adopted for the chloride concentration a substantial entry of chloride would have been predicted, and this may explain the initial rise in chloride concentration observed by Steinbach. The difference between the theoretical rates for sodium and potassium and those observed by Steinbach & Spiegelman is not larger than would be expected from the nature of the calculations used in making the comparison. However, a difference of this kind is to be expected, since it is likely that entry of sodium would be partly compensated by the active extrusion process normally responsible for maintaining a low internal sodium concentration in the living animal.

The experiments described in this paper suggest that sodium ions enter the axon during the rising phase of the spike, and that the rate of rise is determined by the speed at which the charge on the membrane capacity is altered by entry of sodium. It is natural to inquire how large the sodium permeability would have to be in order to give a rate of rise comparable to that observed

experimentally. The problem may be formulated in a different way. The maximum rate of rise of the spike is of the order of 600 V./sec. and, for a membrane capacity of 1·5 μF.cm.$^{-2}$, this corresponds to an ionic current density of 0·9 mA.cm.$^{-2}$. The maximum rate occurs approximately at zero membrane potential, and we may suppose that at this moment the permeability coefficients have already assumed their fully active values of $P_K : P_{Na} : P_{Cl} = 1:20:0{\cdot}45$. We are also given the fact that the resistance of the resting membrane is roughly 1000 Ω.cm.^{2}, and in this condition we assume as before that $P_K : P_{Na} : P_{Cl} = 1:0{\cdot}04:0{\cdot}45$. This information allows the total ionic current to be calculated by the methods described in the Appendix. We find

(1) an inward sodium current of 1·3 mA.cm.$^{-2}$;
(2) an outward potassium current of 0·06 mA.cm.$^{-2}$;
(3) an outward chloride current of 0·04 mA.cm.$^{-2}$;
(4) a net inward current of 1·2 mA.cm.$^{-2}$.

The total inward current is of the same order as that obtained experimentally so that there is no difficulty in accounting for the rate of rise of the action potential in terms of our hypothesis.

The preceding calculation suggests that the inward sodium current greatly exceeds the outward potassium current during the rising phase of the action potential, and we should expect that this situation would be reversed during the falling phase of the spike. A minimum value for the quantity of sodium entering the axon can be obtained by assuming that the period of sodium entry does not overlap to any appreciable extent with the period of potassium exit. In this case the total quantity of sodium entering the axon would be given by the product of the membrane action potential and the membrane capacity divided by the Faraday. Thus $1{\cdot}5 \times 10^{-12}$ mol. must be transferred through a membrane of capacity 1·5 μF. in order to change its potential difference from +50 mV. to −45 mV. More sodium would enter if there was a simultaneous exchange of potassium and sodium, but the quantity entering could not be less than $1{\cdot}5 \times 10^{-12}$ mol. unless some other mechanism assists in the active process. A crucial test of the sodium hypothesis would therefore be to measure the quantity of sodium entering the axon in one impulse. This experiment has never been performed in a satisfactory way, although the work of Fenn & Cobb (1936), Tipton (1938) and v. Euler, v. Euler & Hevesy (1946) provides some indication of sodium entry during activity. The total charge moving out through the membrane during the falling phase must be approximately equal to the charge transferred during the rising phase. The outward charge would be carried primarily by potassium ions if the permeability of the active membrane is greater to potassium than to chloride. Under these conditions a minimum potassium leakage of $1{\cdot}5 \times 10^{-12}$ mol. is to be expected. This is not far from the value obtained by Hodgkin & Huxley (1947), who gave an average value of

$1{\cdot}7 \times 10^{-12}$ moles in *Carcinus* axons with an average membrane capacity of $1{\cdot}3\ \mu$F.cm.$^{-2}$. The average action potential in *Carcinus* axons has been estimated at about 120 mV. (Hodgkin, 1947) so that the theoretical minimum for the potassium leakage would be

$$\frac{120 \text{ mV.} \times 1{\cdot}3\ \mu\text{F.cm.}^{-2}}{96{,}500 \text{ coulomb mol.}^{-1}} = 1{\cdot}6 \times 10^{-12} \text{ mol.cm.}^{-2}.$$

The close agreement is unlikely to be more than a coincidence, but the similarity in order of magnitude may be significant, since Keynes (1948) has recently obtained comparable results by the use of radioactive tracers.

SUMMARY

The reversal of membrane potential during the action potential can be explained if it is assumed that the permeability conditions of the membrane in the active state are the reverse of those in the resting state. The resting membrane is taken to be more permeable to potassium than sodium, and the active membrane more permeable to sodium than to potassium. (It is suggested that the reversal of permeability is brought about by a large increase in sodium permeability and that the potassium permeability remains unaltered or undergoes a relatively small change.) A reversed membrane potential can arise in a system of this kind if the concentration of sodium in the external solution is greater than that in the axoplasm.

This hypothesis is supported by the following observations made with a microelectrode in squid giant axons:

1. The action potential is abolished by sodium-free solutions, but returns to its former value when sea water is replaced.

2. Dilution of sea water with isotonic dextrose produces a slight increase in resting potential, but a large and reversible decrease in the height of the action potential. The reversed potential difference of the active membrane depends upon the sodium concentration in the external fluid and is reduced to zero by solutions containing less than about 30% of the normal sodium concentration.

3. The height of the action potential is increased by a hypertonic solution containing additional sodium chloride, but is not increased by addition of dextrose to sea water. The resting potential is unaffected or slightly reduced by sodium-rich solutions.

4. The changes in active membrane potential which result from increases or decreases of external sodium are of the same order of magnitude as those for a sodium electrode.

5. The rate of rise of the action potential can be increased to 140% of its normal value and reduced to 10% by altering the concentration of sodium in

the external solution. To a first approximation, the rate of rise is directly proportional to the external concentration of sodium.

6. The conduction velocity undergoes a substantial decrease in solutions of low-sodium content.

7. The changes produced by dilution of sea water with isotonic dextrose appear to be caused by reduction of the sodium concentration and not by alterations in the concentrations of other ions.

Removal of external potassium causes a small increase in action potential which is almost entirely due to an increase in the resting potential, the reversed potential difference of the active membrane remaining substantially constant. Increasing the external potassium causes a depression of both action potential and resting potential, but the former is affected to a much greater extent than the latter. The positive phase of the squid action potential is markedly increased by potassium-free solutions and decreased by potassium-rich solutions.

The effects of a large number of solutions on the membrane potential in the resting, active and refractory state are shown to be consistent with a quantitative formulation of the sodium hypothesis.

We wish to express our gratitude to the Rockefeller Foundation for Medical Research for financial aid; to the director and staff of the Laboratory of the Marine Biological Association, Plymouth, for their assistance at all stages of the experimental work; and to Mr A. F. Huxley for much helpful and stimulating discussion.

APPENDIX

This section contains a brief description of the way in which constant field equations may be derived and applied to practical problems. The treatment is essentially similar to that of Goldman (1943) but is summarized here for the convenience of the reader.

The basic assumptions are (1) that ions in the membrane move under the influence of diffusion and the electric field in a manner which is essentially similar to that in free solution; (2) that the electric field may be regarded as constant throughout the membrane; (3) that the concentrations of ions at the edges of the membrane are directly proportional to those in the aqueous solutions bounding the membrane; and (4) that the membrane is homogeneous.

Assumption (1) leads to the following equations for the current carried by ions:

$$-I_{\mathrm{K}} = RTu_{\mathrm{K}}\frac{dC_{\mathrm{K}}}{dx} + C_{\mathrm{K}}u_{\mathrm{K}}F\frac{d\psi}{dx}, \tag{1·1}$$

$$-I_{\mathrm{Na}} = RTu_{\mathrm{Na}}\frac{dC_{\mathrm{Na}}}{dx} + C_{\mathrm{Na}}u_{\mathrm{Na}}F\frac{d\psi}{dx}, \tag{1·2}$$

$$-I_{\mathrm{Cl}} = -RTu_{\mathrm{Cl}}\frac{dC_{\mathrm{Cl}}}{dx} + C_{\mathrm{Cl}}u_{\mathrm{Cl}}F\frac{d\psi}{dx}. \tag{1·3}$$

Here I_{K}, I_{Na} and I_{Cl} are the contributions of potassium, sodium and chloride to the total inward current density through the membrane. C_{K}, C_{Na} and C_{Cl} are the concentrations of ions in the membrane and u_{K}, u_{Na} and u_{Cl} are their mobilities; x is the distance through the membrane from the outer boundary defined as $x=0$. The inner boundary is defined as $x=a$. ψ is the potential

at a point x; R, T and F have their usual significance. In the steady state I_K, I_{Na} and I_{Cl} must be constant throughout the membrane; $d\psi/dx$ is also regarded as constant and equal to $-V/a$, where V is the potential of the outside solution minus that of the inside solution. Equations (1·1), (1·2) and (1·3) may therefore be integrated directly. Thus (1·1) gives

$$\left[\frac{aI_K e^{-VFx/RTa}}{VFu_K}\right]_0^a = \left[C_K e^{-VFx/RTa}\right]_0^a. \tag{2·1}$$

Hence
$$I_K = \frac{u_K FV}{a}\,\frac{(C_K)_o - (C_K)_a e^{-VF/RT}}{1 - e^{-VF/RT}}. \tag{2·2}$$

Now the concentration $(C_K)_o$ at the outer edge of the membrane is regarded as directly proportional to the concentration $(K)_o$ of potassium in the external fluid. Hence

$$(C_K)_o = \beta_K\,(\mathrm{K})_o \quad \text{and} \quad (C_K)_a = \beta_K\,(\mathrm{K})_i,$$

where β_K is the partition coefficient between the membrane and the aqueous solution; $(\mathrm{K})_i$ is the concentration in the axoplasm.

Equation (2·2) then becomes

$$I_K = P_K\,\frac{F^2 V}{RT}\,\frac{(\mathrm{K})_o - (\mathrm{K})_i\,e^{-VF/RT}}{1 - e^{-VF/RT}}, \tag{2·3}$$

where P_K is a permeability coefficient defined as $u_K\,\beta_K\,RT/aF$.

In a similar way we obtain

$$I_{Na} = P_{Na}\,\frac{F^2 V}{RT}\,\frac{(\mathrm{Na})_o - (\mathrm{Na})_i\,e^{-VF/RT}}{1 - e^{-VF/RT}}, \tag{2·4}$$

and
$$I_{Cl} = P_{Cl}\,\frac{F^2 V}{RT}\,\frac{(\mathrm{Cl})_i - (\mathrm{Cl})_o\,e^{-VF/RT}}{1 - e^{-VF/RT}}. \tag{2·5}$$

The total ionic current density through the membrane is therefore given by

$$I = \frac{F^2 V P_K}{RT}\,\frac{w - ye^{-VF/RT}}{1 - e^{-VF/RT}}, \tag{3·0}$$

where
$$w = (\mathrm{K})_o + \frac{P_{Na}}{P_K}(\mathrm{Na})_o + \frac{P_{Cl}}{P_K}(\mathrm{Cl})_i,$$

$$y = (\mathrm{K})_i + \frac{P_{Na}}{P_K}(\mathrm{Na})_i + \frac{P_{Cl}}{P_K}(\mathrm{Cl})_o.$$

The potential difference across the membrane in the absence of ionic current will be designated E. $V = E$ when $I = 0$. Hence

$$E = \frac{RT}{F}\log_e\frac{y}{w}, \tag{4·0}$$

which is equivalent to equation (4) used in the text. The membrane conductance G is defined as $(dI/dV)_{I\to 0}$ and is given by

$$G = \frac{F^2 P_K}{RT}\left\{V\frac{d}{dV}\left[\frac{w - ye^{-VF/RT}}{1 - e^{-VF/RT}}\right] + \left[\frac{w - ye^{-VF/RT}}{1 - e^{-VF/RT}}\right]\right\}. \tag{5·0}$$

The second term in this expression is zero when $I = 0$ and $V = E$. After differentiation V may be equated to E. Hence

$$G = \frac{F^3}{(RT)^2}\,EP_K\left\{\frac{wy}{y-w}\right\}. \tag{6·0}$$

This expression allows us to compute the numerical values of the permeability coefficient P_K provided that the ratios P_{Na}/P_K and P_{Cl}/P_K are known. For the case considered in the text P_K is found to be $1{\cdot}8 \times 10^{-6}$ cm.sec.$^{-1}$. The individual ionic currents may be determined by using this value in applying equations (2·3), (2·4), and (2·5) to any particular set of experimental conditions.

When $I=0$ and $V=E$ a more convenient method is to use the following relations which may be obtained from (2·3), (2·4), (2·5), (4·0) and (6·0):

$$I_K=\frac{RT}{F}G\left\{\frac{(\mathrm{K})_o}{w}-\frac{(\mathrm{K})_i}{y}\right\}, \tag{7·0}$$

$$I_{Na}=\frac{RT}{F}G\frac{P_{Na}}{P_K}\left\{\frac{(\mathrm{Na})_o}{w}-\frac{(\mathrm{Na})_i}{y}\right\}, \tag{7·1}$$

$$I_{Cl}=\frac{RT}{F}G\frac{P_{Cl}}{P_K}\left\{\frac{(\mathrm{Cl})_i}{w}-\frac{(\mathrm{Cl})_o}{y}\right\}. \tag{7·2}$$

These equations were used in the calculation given on p. 71.

The constant field equations may be applied to the rising phase of the spike if it is assumed that the rate of change of potential is low enough to allow the ionic currents to attain their steady state value. At the moment when the rate of rise of the spike is at a maximum the total membrane current is zero, but there is a large ionic current which is equal and opposite to the capacity current through the membrane dielectric. In this case we cannot use (7·0), (7·1) and (7·2), but must return to (2·3), (2·4) and (2·5). Since the maximum rate of rise occurs at approximately the time when $V=0$ these equations may be simplified to

$$I_K=P_K F\,[(\mathrm{K})_o-(\mathrm{K})_i], \tag{8·0}$$

$$I_{Na}=P_{Na} F\,[(\mathrm{Na})_o-(\mathrm{Na})_i], \tag{8·1}$$

$$I_{Cl}=P_{Cl} F\,[(\mathrm{Cl})_i-(\mathrm{Cl})_o]. \tag{8·2}$$

In making the calculation on p. 72 we assumed that when $V=0$, P_K and P_{Cl} had the same values as in the resting nerve, but that P_{Na} was $20P_K$ instead of $0{\cdot}04P_K$.

REFERENCES

Arvanitaki, A. & Chalazonitis, N. (1947). *Arch. int. Physiol.* **54**, 406.
Bernstein, J. (1912). *Electrobiologie*. Braunschweig: Vieweg.
Bishop, G. H. (1932). *J. cell. comp. Physiol.* **1**, 177.
Cole, K. S. & Curtis, H. J. (1938). *J. gen. Physiol.* **21**, 757.
Cole, K. S. & Curtis, H. J. (1939). *J. gen. Physiol.* **22**, 649.
Cole, K. S. & Hodgkin, A. L. (1939). *J. gen. Physiol.* **22**, 671.
Curtis, H. J. & Cole, K. S. (1942). *J. cell. comp. Physiol.* **19**, 135.
Davson, H. (1940). *J. cell. comp. Physiol.* **15**, 317.
Davson, H. & Reiner, M. (1942). *J. cell. comp. Physiol.* **20**, 325.
Erlanger, J. & Blair, E. A. (1938). *Amer. J. Physiol.* **124**, 341.
Fenn, W. O. & Cobb, D. M. (1936). *Amer. J. Physiol.* **115**, 345.
Gallego, A. & Lorente de Nó (1947). *J. cell. comp. Physiol.* **29**, 189.
Gerard, R. W. (1932). *Physiol. Rev.* **12**, 469.
Glazebrook, Sir R. (1923). *A Dictionary of Applied Physics*, vol. 3. London: Macmillan.
Goldman, D. E. (1943). *J. gen. Physiol.* **27**, 37.
Grundfest, H. (1947). *Ann. Rev. Physiol.* **9**, 477.
Henderson, P. (1907). *Z. phys. Chem.* **59**, 118.
Henderson, P. (1908). *Z. phys. Chem.* **63**, 325.
Höber, R. (1946). *Ann. N.Y. Acad. Sci.* **47**, 381.
Hodgkin, A. L. (1939). *J. Physiol.* **94**, 560.
Hodgkin, A. L. (1947). *J. Physiol.* **106**, 305.
Hodgkin, A. L. (1948). *J. Physiol.* **107**, 165.
Hodgkin, A. L. & Huxley, A. F. (1939). *Nature, Lond.*, **144**, 710.
Hodgkin, A. L. & Huxley, A. F. (1945). *J. Physiol.* **104**, 176.
Hodgkin, A. L. & Huxley, A. F. (1947). *J. Physiol.* **106**, 341.

Kato, G. (1936). *Cold. Spr. Harb. Symp. quant. Biol.* **4**, 202.
Katz, B. (1947). *J. Physiol.* **106**, 411.
Keynes, R. D. (1948). *J. Physiol.* **107**, 35 *P*.
Krogh, A. (1946). *Proc. Roy. Soc.* B, **133**, 123.
Landolt-Börnstein (1931). *Physicalisch-chemische Tabellen*, 5th ed. Ergänzungsband IIa, 189. Berlin: Springer.
Lillie, R. S. (1923). *Protoplasmic Action and Nervous Action.* Chicago: University Press.
Lorente de Nó, R. (1944). *J. cell. comp. Physiol.* **24**, 85.
Lorente de Nó, R. (1947). *A Study of Nerve Physiology*, vols. 1 and 2 in *Studies from the Rockefeller Institute for Medical Research*, vols. **131** and **132**. New York.
Overton, E. (1902). *Pflüg. Arch. ges. Physiol.* **92**, 346.
Planck, M. (1890 *a*). *Ann. Phys., Lpz.*, **39**, 161.
Planck, M. (1890 *b*). *Ann. Phys., Lpz.*, **40**, 561.
Schmitt, F. O. (1930). *Amer. J. Physiol.* **95**, 650.
Schmitt, F. O. & Schmitt, O. H. A. (1931). *Amer. J. Physiol.* **97**, 302.
Steinbach, H. B. (1941). *J. cell. comp. Physiol.* **17**, 57.
Steinbach, H. B. & Spiegelman, S. (1943). *J. cell. comp. Physiol.* **22**, 187.
Stella, G. (1928). *J. Physiol.* **66**, 19.
Tipton, S. R. (1938). *Amer. J. Physiol.* **124**, 322.
Ussing, H. H. (1947). *Nature, Lond.*, **160**, 262.
v. Euler, H. V., v. Euler, U. S. & Hevesy, G. (1946). *Acta physiol. Scand.* **12**, 261.

J. Physiol. (1949) 108, 78–91 612.843.4

INTENSITY DISCRIMINATION OF THE CENTRAL FOVEA MEASURED WITH SMALL FIELDS

By L. C. THOMSON

From the Vision Research Unit, Medical Research Council, London

(*Received* 15 *January* 1948)

By means of a small test field, subtending 15′ at the eye, some information on the shape of the energy-response curve for the fovea has been obtained and reported in a previous paper (Thomson, 1947). This curve records the relationship between the retinal response and the amount of light energy applied to the receptors. It was found that the slope of the curve was not the same for all wave-lengths; in fact, red energy had to be increased by a larger factor than had blue-green energy in order to achieve the same increase in the response. Thus the slope of the blue-green was much steeper than that of the red curve (Thomson, 1947, Fig. 9). It would seem that the ability to discriminate between one intensity and another slightly higher one must rest to some extent upon the slope of these curves. Suppose a just noticeable increase in the response was the same for every wave-length, then, since the slope of the red curves is relatively less steep than that for other wave-lengths, one might expect that a larger increment of energy would be necessary to elicit a just noticeable difference of brightness at red wave-lengths.

Thus an investigation, with the small field method, of the energy increment necessary to evoke a just noticeable difference of brightness at various wave-lengths might provide corroboration for the shape of the energy-response curves and possibly other data of interest about the behaviour of the fovea to increasing amounts of light energy. Actually the results have been expressed as the logarithm of the fraction, $\Delta I/I$, and not as the energy increment itself, ΔI.

Some experiments were also made with the small field displaced by 40′ from the fixation point into the left visual field.

METHOD

The measurements were made with the Wright colorimeter at Imperial College (Wright, 1946), and the field size was identical with that used in a previous investigation (Thomson & Wright, 1947). Briefly it consisted of a small circular aperture which was divided horizontally into an upper test and lower comparison field, each semicircular and of approximately equal area. The wave-length and intensity of the light illuminating each half was variable. The field was seen upon a dark surround.

For the observations at 40′ a small red fixation spot was introduced in an appropriate position and maintained at a brightness similar to that of the main field. Some observations were also made with a divided field arrangement as shown in the insert to Fig. 5. In these measurements the lower comparison field remained at the fixation point, and the upper test field only was displaced by the required amount into the left visual field. Each figure in this paper contains an insert showing the type of field used.

Procedure. The wave-length of the light illuminating each half of the field was made equal and a series of between 10 and 15 (for most wave-lengths) arbitrary intensity values were chosen for the lower field which would cover evenly the whole brightness range at that wave-length from near the threshold of vision to the maximum attainable in the colorimeter; a range of intensity for most wave-lengths of about $\times 10^4$ when a small field is used at the foveal centre. Then, having set the lower field to the lowest of these chosen values, the observer (L.C.T., right eye) made a pair of brightness matches between the two fields. The readings of the neutral wedge controlling the intensity of the upper field were recorded, and the upper field intensity was then set so that a just noticeable increase in brightness could be detected. A further record was made and the just noticeable brightness step repeated, so that for each setting of the lower field intensity four readings of the neutral wedge were available: two 'matches', from which the intensity of the lower field, I, could be calculated and two 'steps', which would yield $I+\Delta I$, where ΔI is the increase in I required to produce a just noticeable difference of brightness. The next higher arbitrary intensity value was then set in the lower field and the four recordings repeated. Each wave-length setting was completed without a break, and five was the usual number of runs for any wave-length, each performed at a different observing session. Four wave-length settings were sufficient for one session, and those four were completed before doing any others. Thus a whole family of 22 wave-length curves was covered in groups of four, each member of the group being chosen from a different part of the spectrum.

Calculation. From the average wedge readings for I and $I+\Delta I$ at each intensity value of the lower field the amount of energy from each half of the field, which passed through the exit pupil of the colorimeter (diameter 2 mm.), was calculated. ΔI was then determined by subtraction and the result expressed as $\log \Delta I/I$, which values are the ordinates in the diagrams of this paper. The abscissae in the diagrams are usually the logarithms of the intensity I expressed in 'threshold' units, i.e. the amount of energy required to reach the threshold of vision at any wave-length was considered as unit energy for that wave-length. For this purpose a new and more complete determination of the threshold energy values for L.C.T. was undertaken in September 1947.

Conversion of the results. The values of $\log I$ in threshold units may be converted to the logarithms of the amount of energy from each semicircular field, which passed the exit pupil of the colorimeter, by adding the log threshold energy (log erg/sec.) given for each wave-length in Table 1. The resulting values may be converted into equivalent foot-candles (as seen through a pupil of diameter 2 mm.) by subtracting the further logarithm of $\bar{5}{\cdot}143$. To convert the log erg/sec. values to millilamberts (pupil diameter 2 mm.) substract log $\bar{6}{\cdot}111$.

TABLE 1. Energy (erg/sec.) required at each wave-length and for the two field positions to reach the threshold of vision

μ.	Centre	40′	μ.	Centre	40′
0·44	$\bar{6}{\cdot}058$	$\bar{7}{\cdot}640$	0·56	$\bar{8}{\cdot}219$	$\bar{8}{\cdot}097$
0·45	$\bar{7}{\cdot}898$	$\bar{7}{\cdot}502$	0·57	$\bar{8}{\cdot}208$	$\bar{8}{\cdot}129$
0·46	$\bar{7}{\cdot}678$	$\bar{7}{\cdot}356$	0·58	$\bar{8}{\cdot}228$	$\bar{8}{\cdot}154$
0·47	$\bar{7}{\cdot}443$	$\bar{7}{\cdot}101$	0·59	$\bar{8}{\cdot}345$	$\bar{8}{\cdot}238$
0·48	$\bar{7}{\cdot}364$	$\bar{8}{\cdot}971$	0·60	$\bar{8}{\cdot}384$	$\bar{8}{\cdot}235$
0·49	$\bar{7}{\cdot}273$	$\bar{8}{\cdot}931$	0·61	$\bar{8}{\cdot}459$	$\bar{8}{\cdot}284$
0·50	$\bar{8}{\cdot}992$	$\bar{8}{\cdot}630$	0·62	$\bar{8}{\cdot}567$	$\bar{8}{\cdot}384$
0·51	$\bar{8}{\cdot}753$	$\bar{8}{\cdot}517$	0·63	$\bar{8}{\cdot}730$	$\bar{8}{\cdot}553$
0·52	$\bar{8}{\cdot}636$	$\bar{8}{\cdot}438$	0·64	$\bar{8}{\cdot}892$	$\bar{8}{\cdot}721$
0·53	$\bar{8}{\cdot}370$	$\bar{8}{\cdot}221$	0·65	$\bar{7}{\cdot}043$	$\bar{8}{\cdot}873$
0·54	$\bar{8}{\cdot}249$	$\bar{8}{\cdot}149$	0·66	$\bar{7}{\cdot}203$	$\bar{7}{\cdot}036$
0·55	$\bar{8}{\cdot}220$	$\bar{8}{\cdot}088$			

Precision. At each of several I values the standard error of the mean of five estimates of $\Delta I/I$ has been calculated according to the formula $\sqrt{\{\Sigma (x-\bar{x})^2\}/n(n-1)}$, where x is any value of $\Delta I/I$, $\bar{x}$ the mean of the five estimates, and n the number of the estimates (five in most cases). To do this the colorimeter readings were not averaged at once, but each was expressed in energy units and each $\Delta I/I$ worked out separately. The mean so obtained did not differ very greatly from that calculated by the usual method, and although the calculation in this paragraph is strictly the more correct procedure, the labour involved prohibited its use for all readings. The points at which errors have been calculated are shown in the figures by a vertical line indicating a range of ordinate values ot $\pm\sigma_{\text{mean}}$. The errors in the abscissae are very small in comparison and have been omitted. By estimating the standard deviation of the mean from a sample of five, the estimate itself is bound to have a moderately large error of its own, and it should be borne in mind when looking at the figures that on some occasions an error line might be one-third longer than shown.

Criterion. The criterion of discrimination used in this investigation is the just noticeable difference in brightness, and during the measurements the ΔI required to elicit a just noticeable difference of brightness did not always remain the same under similar conditions. It appeared that the criterion would remain moderately constant for many weeks during an unbroken series of sessions, but that if a rest was taken and a further series started, the criterion might be somewhat different. It was the usual practice to leave the results of any series unexamined until it was complete. This step seemed to lead to a stable just noticeable difference within that series. Further, stability was much increased as soon as the novelty of the experiment had worn off, and towards the end, when the experiment had become automatic, the colorimeter could be set whilst thinking of other things and the stability of the just noticeable difference was then at its best. Fluctuations of the criterion did not at any time influence the size of $\Delta I/I$ by more than 15%.

Calibration of the colorimeter. The absolute brightness calibration of the colorimeter rests upon a comparison (using a 1° 20′ field) between the spectral light of 0·520 μ. and the white light from a standard lamp, standardized at the National Physical Laboratory, using a method similar to that described in a previous paper (Thomson, 1947).

The calibration of the relative amounts of energy at each wave-length depends upon new measurements made as follows. A ribbon filament lamp was calibrated for colour temperature against a lamp of known colour temperature (2848° K), which had been standardized at the National Physical Laboratory. The light from this lamp was then refracted into a spectrum with a simple auxiliary spectroscope, and the light passing through the exit slit allowed to fall on the cathode of a multiplier photocell. The output from the photocell was measured with a Cambridge Unipivot galvanometer and the deflexions were recorded for each 10 mμ. of the spectrum. The light passing the exit pupil of the Wright colorimeter was then allowed to illuminate the cathode and the neutral wedge controlling the intensity so adjusted that, for any wave-length, the galvanometer deflexion was similar to that obtained at the same wave-length in the auxiliary spectroscope. The readings of the neutral wedge were recorded. Thus, after the relative energy values at each wave-length for the calibrated lamp had been weighted by a correction for the exit slit width of the auxiliary spectroscope, the amount of energy corresponding to a neutral wedge reading was known at each wave-length. Losses of light in the glass parts of the auxiliary spectroscope were neglected. Since the final calibration between wave-lengths 0·500 and 0·660 μ. agreed to within 12% with the original calibration performed with a thermojunction, and since this agreement was obtained in spite of the fact that a different colorimeter lamp was in use, the calibration as a whole was considered satisfactory.

RESULTS

Central area. The results for the centre of the central fovea are shown in Fig. 1, and the field arrangement was as shown in the insert. Each set of points corresponds to a different wave-length setting, which is shown in μ. on the face of the figure, and since each, if plotted on the same ordinate scale, overlies the next, the scale of log $\Delta I/I$ is correct for wave-length 0·660 μ. only. Each scale

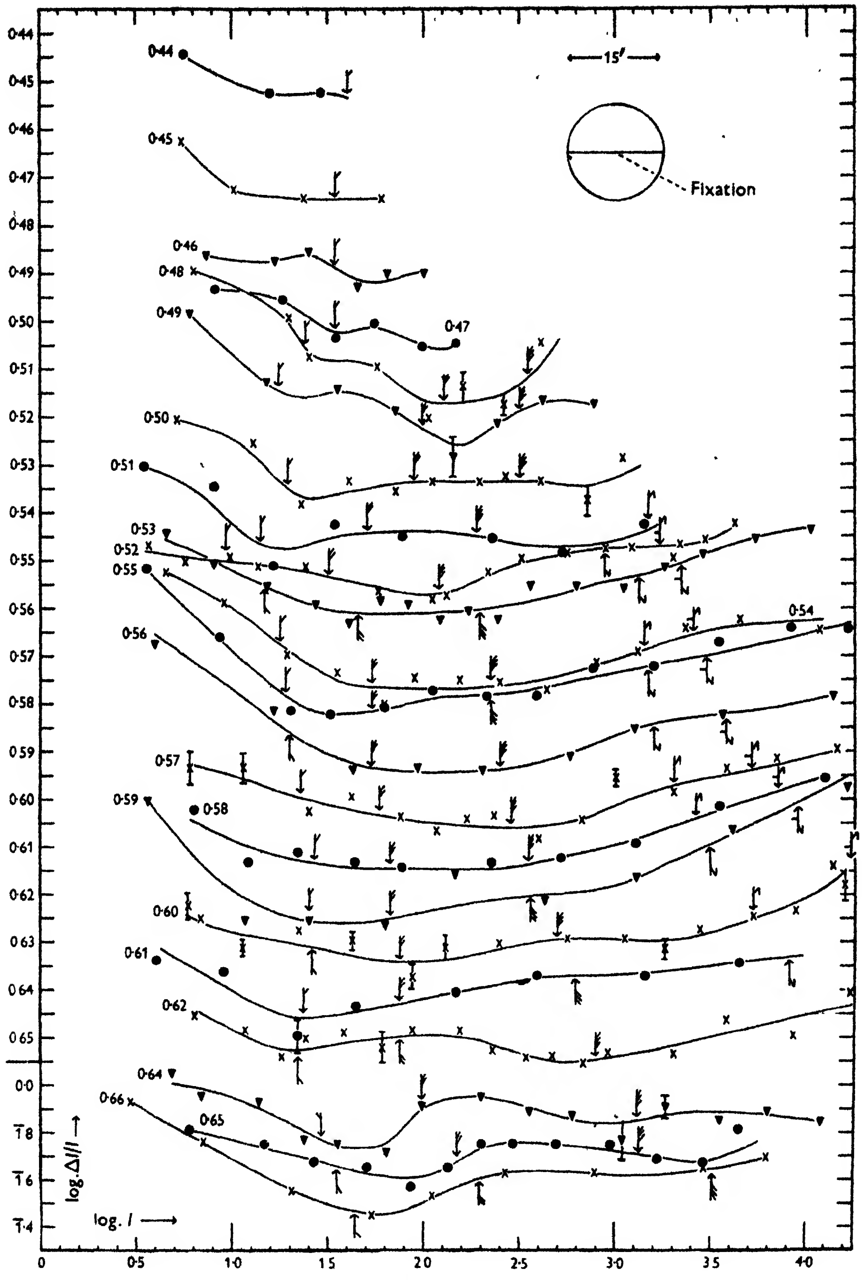


Fig. 1. Intensity discrimination of the centre of the central fovea for twenty-two wave-length settings. Insert shows the type of field used. Ordinate scale correct for wave-length 0·660 μ. only; each other scale displaced upwards from the next by 0·2 log unit, the value 0·0 for any scale being shown by the value of the wave-length in μ. on the ordinate. Curves are freehand. The arrows indicate equi-brightness levels. level 1, level 2, level 3, level 4, level 5.

has been displaced upwards from its neighbour by 0·2 log unit and the value 0·0 for any wave-length is indicated by writing the value of that wave-length in μ. upon the ordinate. The curves drawn through the points are freehand to be a good fit to the data and do not represent any mathematical function.

Wave-lengths 0·450, 0·510, 0·560, 0·580, 0·590 and 0·610 μ. were all performed during the first few weeks of 1947, and, when first plotted on the same ordinate scale, showed a general increase in the size of $\Delta I/I$ upon that obtained at other wave-lengths, which were all completed before Christmas, 1946; an increase which was most probably due to an altered criterion. Hence the value of $I+\Delta I$ in these six curves has been lowered by 7·2 % throughout in an attempt to allow for this change. A check on the validity of this adjustment will be mentioned below. From a previous investigation (Thomson, 1947) the amount of energy at each wave-length required to stimulate a sensation of equal brightness was known for this observer and for five brightness levels under similar observing conditions. The five levels have been marked on Fig. 1 with distinctive arrows. The curves divide into a low-intensity part, in which the discrimination is worsening as the intensity is lowered ($\Delta I/I$ increasing); a middle part in which $\Delta I/I$ is approximately constant; and an upper part, in which discrimination is again falling off as the intensity rises and the field becomes glaring in brightness. The degree to which discrimination falls off in the lower part varies with wave-length, and this is strikingly shown at wave-lengths 0·480, 0·470 μ. and in the region of 0·520 μ. At 0·480 μ. the decrease is so great that the curve for this wave-length has crossed that for 0·470 μ. even though they are as a whole displaced from each other by 0·2 log unit. A similar, but not so marked, effect is seen in the high-intensity region between wave-lengths 0·570 and 0·590 μ.

Another feature is the large wave seen in the curves for red wave-lengths near brightness level 2. This wave is due to a disproportionate increase in the size of ΔI at this brightness level, and since it is at just this level that the red energy-response curves reported previously (Thomson, 1947, Fig. 9) diverge from their fellows for other wave-lengths, it seems that the energy-response curves do influence intensity discrimination to some extent. However, the disproportionate increase in ΔI is not maintained at higher brightnesses as one would expect from an examination of the energy-response curves. In addition, the red wave does not appear in any curve for wave-lengths shorter than 0·620 μ. even though the energy-response curves for several wave-lengths shorter than this show a decrease in slope, so that agreement between the two investigations is by no means perfect.

Several wave-lengths show a small depression near brightness level 1. This is well shown between wave-lengths 0·460 and 0·510 μ. and again between 0·610 and 0·660 μ. Experimental error accounts for neither this depression nor

the 'red wave', already mentioned. The high-intensity portion of the curve seems to begin either at or slightly above level 3.

Equi-brightness section. It is possible graphically to determine from the smooth freehand curves the value of log $\Delta I/I$ for each wave-length at each of

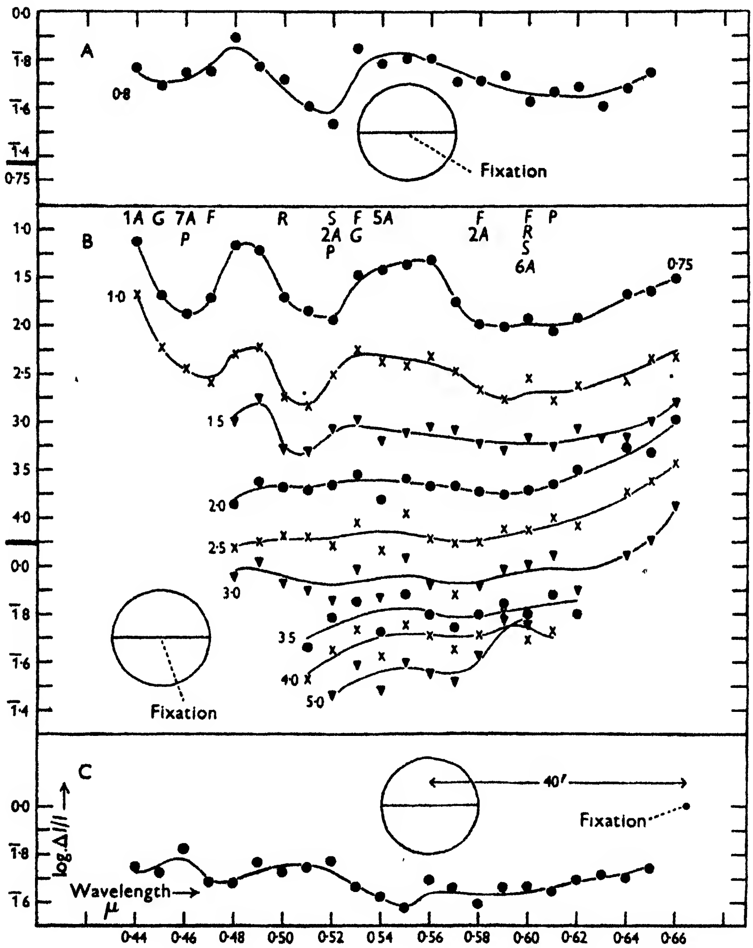


Fig. 2. Intensity discrimination of the fovea shown as a function of wave-length at equal brightness. Ordinate scale displaced as in Fig. 1. The letters in section B indicate the positions in the spectrum of maximum modulator activity as found by Granit. *F*, frog; *G*, guinea-pig; *R*, rat; *S*, snake; 2*A*, two maxima found by adaptation; *P*, maximum found by polarization.

the five brightness levels, and in addition at other arbitrary levels: 0·75 between the threshold and level 1; half-way between levels 1 and 2, levels 2 and 3 and levels 3 and 4. The values of log $\Delta I/I$ so obtained can then be plotted on the ordinate against wave-lengths as abscissae, and the result is shown in Fig. 2B.

Here again the ordinate scale has been displaced and is correct for level 5 only. Each other curve is displaced upwards by 0·2 log unit.

The variable slope of the lower parts of the Fig. 1 curves noted earlier has produced at low brightness levels a markedly humped curve with three minima; one in the blue, one in the blue-green and another broader one covering yellow, orange and some red wave-lengths. Since the vertical position of a point on these curves depends upon ΔI, and since all the twenty-two curves were not obtained in the same series of experiments, it might be argued that these minima were due in some way to a variable criterion. In order to meet this criticism a value for I for each wave-length was calculated so that it would have a brightness approximately equal to 0·60 of level 1. Each value was then presented to the observer in random order (so that errors of criterion would be similar for all) and log $\Delta I/I$ obtained in the usual way. The results of five runs are shown in Fig. 2A. At these low brightnesses the observer can only identify a wave-length by its colour in the broadest terms, i.e. reddish, greenish or bluish, and thus identification of a wave-length by its colour does not seriously interfere with the randomization. The points in Fig. 2A are, of course, subject to the full experimental error and were not obtained from smoothed curves. Bearing these facts in mind, the agreement between A and B in Fig. 2 is satisfactory, and thus alteration of the criterion is unlikely to have caused the minima in the data.

At high brightness levels a falling off in discrimination is seen in the region of wave-length 0·590 μ.

40′ *area.* A few wave-length settings were repeated with the field displaced by 40′ into the left visual field and the results are shown in Fig. 3. Here the worsening of discrimination at low brightness can again be seen, but unfortunately the lowest chosen arbitrary value was in most cases not as near the threshold as it might have been. The red wave in the 0·650 μ. curve is well shown, although it appears in these measurements to occur at a lower brightness level than in those for the centre. Wave-lengths 0·440, 0·480, 0·560 and 0·650 μ. are based upon three runs only, and this may partly account for the unusually large error given in the 0·560 μ. series. This error is quite unusual, and was, in fact, the largest of those calculated. For the middle spectral region a very marked decrease of discrimination occurred at high intensities. At the highest point on the 0·560 μ. curve, for instance, ΔI must be some 2·5 times the intensity I for a just noticeable difference to be seen. Brightness difference in its ordinary sense was impossible here, large movements of the intensity control appearing to have little or no effect upon the sensation of brightness.

To perform the measurements a different criterion was used. As the intensity control is operated, the apparent size of the small field changes, being larger for higher and smaller for lower intensities. Thus the two ‘matches’ were really size matches between the two halves of the field and the two ‘steps’, a just

noticeable difference of size. The apparent brightness of the upper field remained amazingly constant in spite of marked size fluctuation, which was, of course, associated with big intensity fluctuation. It seems that at these levels of brightness the receptors have reached a maximum in their brightness response. This effect was absent at blue, violet and red wave-lengths, but this may have been due to limitations in the energy delivery of the colorimeter.

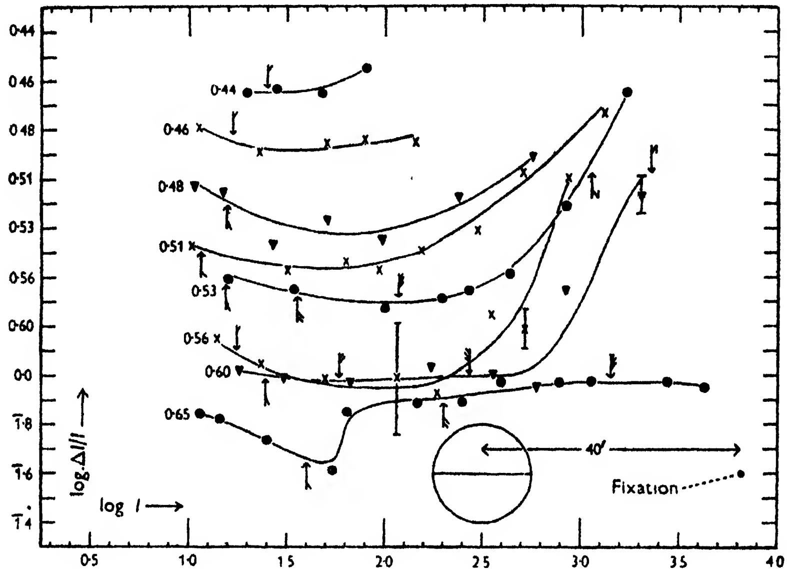


Fig. 3. Intensity discrimination of the fovea at a point displaced by 40′ into the left visual field. Ordinate scale displaced as in Fig. 1.

Discrimination is as a whole poorer here than in the central position. The lower portions of the curves have been investigated more thoroughly and the results are shown in Fig. 4. In order to eliminate differences of criterion the wave-length settings were randomized throughout, and it is apparent that the errors are smaller than in previous series. The figure shows a decided wave in the curves for wave-lengths 0·520–0·560μ., but is otherwise uninteresting. The equi-brightness section at level 1, which is shown in Fig. 2C, is also disappointing.

Divided field arrangement. Some measurements have been made with a divided field arrangement as shown in the insert to Fig. 5. Two wave-length settings only are given. Comparing these results with those in Fig. 3, the presence of the gap between the matching fields at 40′ seems to decrease the power of discrimination still further at most brightness levels, but it is usual to find an improvement towards the lowest intensities. This may be seen in the

20′ curve for wave-length $0{\cdot}530\mu$., but was more striking at other green wave-lengths. The red wave and the marked decrease in discrimination at high brightness are again well shown.

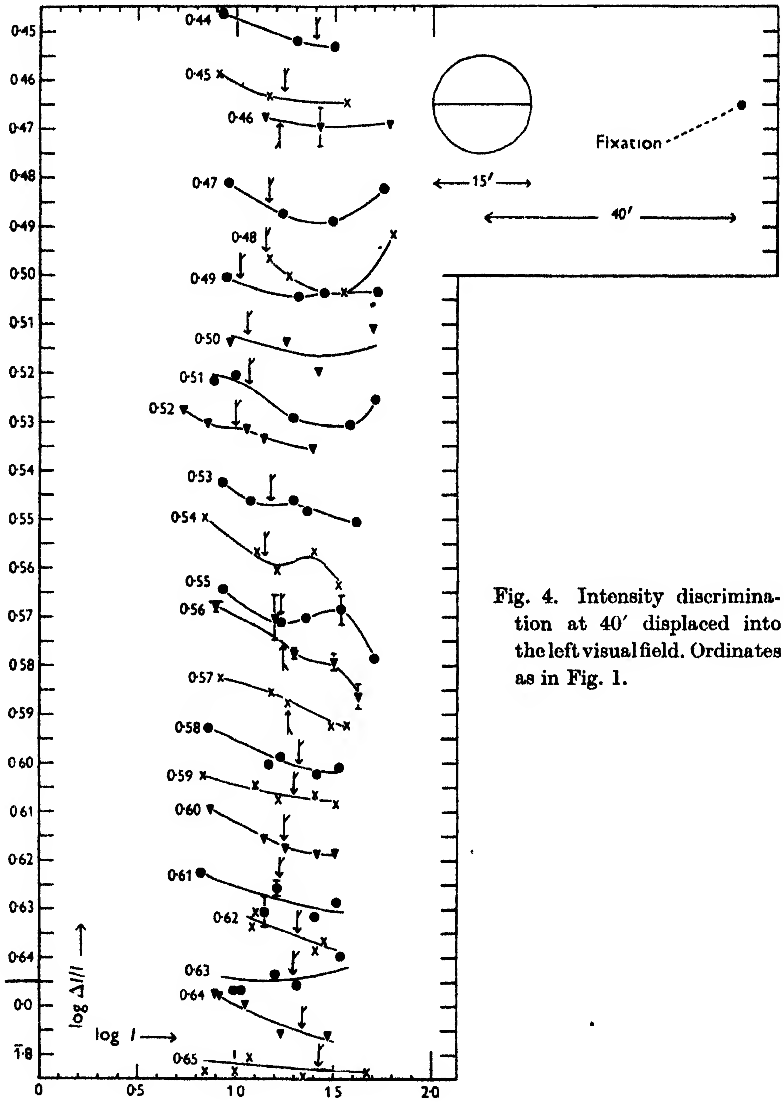


Fig. 4. Intensity discrimination at 40′ displaced into the left visual field. Ordinates as in Fig. 1.

Further phenomena. A careful study of the data in Fig. 1 has led to the tentative suggestion that the curves may show certain other features. If the smoothed $0{\cdot}600\mu$. curve is examined, it is seen to pass outside the error range at three points and to be only just inside this range at three more. Since this range is the standard error of the mean, it is statistically somewhat unlikely

that the smooth curve really fits the points. In addition, one point at wave-length 0·570 μ. between levels 3 and 4 is well off the smooth curve. The experimental error has also been much stretched to obtain a smooth 0·480 μ. curve at high brightness, and there is some indication at several wave-lengths (0·500, 0·510, 0·540, 0·550, 0·570, 0·600, 0·620 and 0·650 μ.) that there is a small dip between brightness levels 3 and 4.

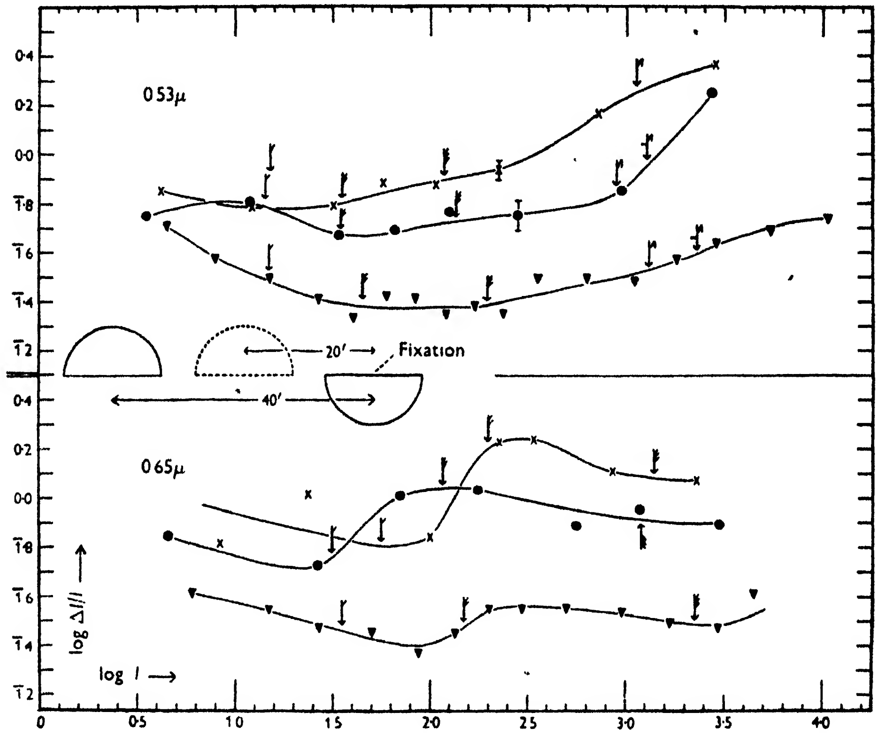


Fig. 5. Intensity discrimination with a divided field arrangement at wave-lengths 0·650 and 0·530 μ. ▼—▼ central; ●—● 20′; ×—× 40′.

In an attempt to elucidate these features a very thorough investigation of wave-length 0·600 μ. was undertaken in December 1947. The whole range of brightness was covered by four runs of 15 points each, so that no two chosen values of the lower field were alike. Each run of 15 points was then performed in the usual way, so that there was no change in the observing conditions. Runs were taken in random order, and the results of the whole series, comprising upwards of 1200 settings of the colorimeter, are plotted with distinctive symbols in Fig. 6A. A smooth curve is shown as a full line and a more complicated function as a dotted one. The question at once arises as to whether this curved function is the result of experimental error or not. The points composing one only of the four runs can scarcely provide an answer, because

the arbitrary choice of the position of the points will sometimes prevent a run from showing all the features. When Fig. 6 A is examined as a whole, however, it does appear to support the curved function better than the smooth. It is the arbitrary choice of points which may account for the somewhat sporadic appearance of the small dip between levels 3 and 4 in Fig. 1. Although the effect is not striking, Fig. 6 A goes some way to showing that the intensity discrimination function in small fields is not a smooth one.

Some corroboration for the curved function has been found in earlier readings. Two series, each of five runs, which were obtained more than a year and a quarter previously are shown in Fig. 6 B. Whilst the two functions have been reproduced without alteration in shape, they have been displaced downwards

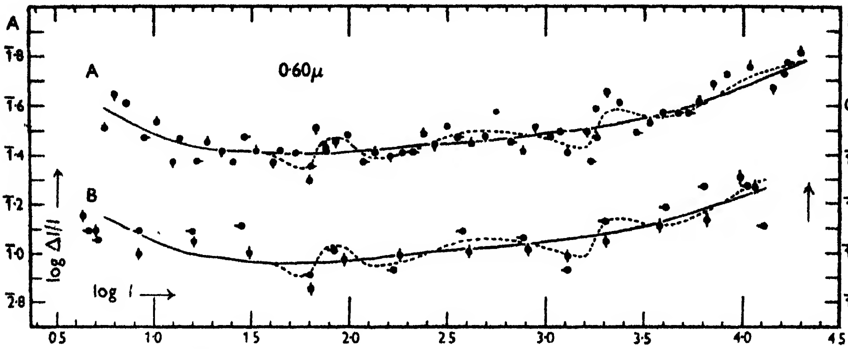


Fig. 6. A thorough investigation of the intensity discrimination of a single wave-length, 0·600 μ. •, ♦, •-, ♦ December 1947; -• July 1946; ♦ October 1946.

by about 12 %. In view of a probable overall change in criterion this is a very small shift. In addition, the points of Fig. 6 B appear to have been uniformly lowered in *I* value, when they are compared with those in Fig. 1. This is due to the use of a different threshold value in Fig. 6 B. The early threshold values as already published (Thomson, 1947) were incomplete, and new values, obtained in September 1947, were used throughout Fig. 1 in the interests of consistency, but in Fig. 6 B the old value is used because it is strictly the more appropriate to these two series given. The agreement over the centre section between these two series and the function obtained from the more recent four runs seems to the author to be too close to be merely due to experimental error. However, agreement is not so good at low intensities, and the curved function is therefore reported as tentative only.

DISCUSSION

Adaptation level of the eye. There are several references in the literature to the need for the eye to be adapted to the intensity *I* before the discrimination measurements are made. It is contended that if this is done,

the discrimination will then continue to improve up to the highest brightness attainable, and that the worsening of discrimination shown in this paper at high brightness is fallacious and is due to inadequate adaptation to the brightness level. However, an investigation in which the adaptation of the eye does not correspond to the brightness of the test patch is not without interest, and in the present experiments the general adaptation of the fovea is probably not far removed from the dark-adapted state even when the highest test field brightnesses are being measured. The reason for this is that a glance technique was used, so that the adapting power of the small test field itself was minimized. The part of the total observing time during which the observer was actually looking at the field was only about 20% of the whole, and this 20% was not all consecutive. Of course the high brightness test fields must have some adaptation effect, but it was felt that the departure from the dark-adapted state was not large. Thus the breakdown of brightness discrimination, noted for green wave-lengths in the 40′ position, indicates a maximum receptor response, when, with the foveal mechanisms nearly dark adapted, a high intensity of stimulation is suddenly presented over a small area.

In practice the onset of a profound state of dark adaptation made the observations difficult. Let us suppose a completely dark visual field was maintained between observations. The retinal lights gradually increased in spite of making further measurements until the clarity of the field was impaired. If, on the other hand, the eyes were directed between observations towards the black wall of the room which was illuminated by stray light from the colorimeter, retinal lights were controlled and observing was much easier. Thus dark adaptation was not complete, but nearly so in these experiments.

Equi-brightness section 0·75. By an electrophysiological method Granit (1947) divides the responses of single-fibre optic nerve preparations into dominator and modulator elements. The dominators have wide spectral sensitivity curves and respond to some extent to most visible wave-lengths. The modulators, on the other hand, are more selective in their response and confine their maximum activity to three main spectral regions.

It is interesting to try to correlate the spectral positions of the minima in the 0·75 equi-brightness section with the maxima of modulator activity found by Granit. The maxima obtained by direct isolation with the micro-electrode technique in the frog were 0·470, 0·530, 0·580 and 0·600 μ.; from the guinea-pig, 0·450 and 0·530 μ.; from the rat, 0·500 and 0·600 μ.; and from the snake, a double-peaked curve with one maximum at 0·600 μ. and the other at 0·520 μ. The spectral positions of these maxima are indicated in Fig. 2 by distinctive letters. More recently, Granit has developed a method in which the micro-electrode technique was combined with selective adaptation of the receptors to coloured light. Modulator maxima were again found when this method was applied to the cat. Of eight curves deduced by this method, two had maxima

at 0·580 μ. and the rest at 0·600 μ. Of seven further curves, five had maxima at 0·540 μ. and two at 0·520 μ. In the blue, seven out of eight curves had maxima at 0·460 μ. and only one at 0·440 μ.

In a recent paper Granit (1948) has used a further new method of investigation. The micro-electrode technique was combined with polarization of the eye by the passing of a current across the optic cup from side to side. Cats were again the experimental animals, and by means of a statistical method, modulator activity has again been demonstrated. The maxima are at wave-lengths 0·460, 0·520 and 0·610 μ. From Fig. 2 it can be seen that the correlation between these maxima and the minima found in the present experiments is, apart from wave-lengths 0·540 and 0·530 μ., remarkably close, and it suggests that the minima are in some way the result of modulator activity.

Why are the discrimination minima not present at higher brightness levels? Granit regards the dominator activity, which, owing to its broad spectral sensitivity response, could be of little use as a means of hue discrimination, as a brightness response, and he reminds us that colour, which may be signalled to the brain by red, green and blue modulators, is to some extent independent of brightness. Both modulators and dominators would, however, contribute to the brightness sensation, but at normal levels this sensation would mainly depend upon dominator activity. Thus intensity discrimination should also depend at these levels upon the dominator response and the value of log $\Delta I/I$ be much the same throughout the spectrum. As the intensity is lowered and the dominator becomes less active, the modulator response would contribute more to the total brightness sensation, and intensity discrimination should therefore tend to become a function of modulator activity and might be expected to show minima at those wave-lengths at which the modulators are most active.

Thus the fact that the discrimination minima do not persist as the brightness is raised can be made to fit into the dominator-modulator idea.

SUMMARY

1. The intensity discrimination of two areas of the foveal retina at twenty-two different wave-lengths has been investigated with a small field (15′).

2 The worsening of discrimination seen at low intensity varies with wave-length, discrimination remaining moderately good at spectral regions in which Granit has found modulator activity.

3. When the field is displaced 40′ into the left visual field, the receptors reach a maximum response for the observing conditions at high brightness, and the relationship between this and the adaptation of the eye is discussed.

Once again my thanks are due to Dr W. D. Wright for his hospitality at Imperial College and also to Miss Gillian Frowde, who has recorded the observations. My thanks are also due to the Medical Research Council for their continued support for visual research at Imperial College and also to Dr H. O. Hartley for his statistical opinion.

REFERENCES

Granit, R. (1947). *Sensory Mechanisms of the Retina.* London: Oxford University Press.
Granit, R. (1948). *J. Neurophysiol.* (in the Press).
Thomson, L. C. (1947). *J. Physiol.* **106**, 368.
Thomson, L. C. & Wright, W. D. (1947). *J. Physiol.* **105**, 316.
Wright, W. D. (1946). *Researches on Normal and Defective Colour Vision.* London: Kimpton.

J. Physiol. (1949) 108, 92–101 612.392.013:612.311.1

THE EFFECTS OF AVITAMINOSIS AND HYPER-VITAMINOSIS A UPON THE INCISOR TEETH AND INCISAL ALVEOLAR BONE OF RATS

By J. T. IRVING

From the Department of Physiology, Medical School, University of Cape Town

(*Received* 19 *January* 1948)

The concept that vitamin A acts on bone formation has been advanced by Mellanby in many publications (e.g. 1938, 1944, 1947), and Wolbach & Bessey (1941) and Wolbach (1946) have also produced theories of a similar nature. Mellanby has reported on the effects of vitamin deficiency and the administration of the vitamin after a period of deficiency. Wolbach and his colleagues have studied both avitaminosis and hypervitaminosis. According to Mellanby (1947), vitamin A governs the action of the osteoblasts and osteoclasts so that bone modelling takes place in an orderly manner. In deficiency of the vitamin, apposition of bone is excessive or may occur on bony faces where usually resorption is found. The osteoblasts and osteoclasts may be reversed in position at the effective surfaces and an orderly disorder occurs. The addition of vitamin A to the diet speedily brings about a return of activity of these cells to the surfaces where it is usually found.

Wolbach (1946) reported that in vitamin A deficiency endochondral bone formation stopped. Appositional bone formation continued until inanition supervened, in strict conformity to the normal growth patterns, both as to situation and to rate, but remodelling sequences, involving concurrent resorption of bone with bone deposition and replacement of cancellous bone by compact bone, ceased.

The toxicity of vitamin A in excessive doses has been investigated by a number of workers, and the literature up to 1945 has been extensively reviewed by Moore & Wang (1945). All writers are agreed that the chief toxic effects are spontaneous fractures of the bones and a tendency to internal haemorrhage. However, many investigators did not consider these actions were due to the vitamin, but to some accompanying impurity. Moore & Wang (1943) were the first to show, by using vitamin A in pure form as a crystalline ester, that the effects of overdosage could indeed be ascribed solely to the vitamin. In further work they confirmed and extended their observations

(1945). Wolbach (1946) reported on the histological changes found in the long bones during hypervitaminosis in rats and guinea-pigs. He concluded that the processes of bone remodelling during growth (epiphyseal cartilage sequences, resorption, and apposition) were greatly accelerated and that the new tissue, hurriedly laid down, was defectively calcified bone or sometimes only osteoid tissue and it fractured easily. van Metre (1947) also found that remodelling processes in the tibia were accelerated in hypervitaminosis A but that longitudinal growth was retarded.

The present paper reports the effects of avitaminosis and hypervitaminosis A on the alveolar bone and upper incisor teeth of rats. Schour, Hoffman & Smith (1941) stated that the socket bone of the incisor teeth appeared thicker than normal with prominent spicules of bone in vitamin A deficiency, and that the growth of the alveolar bone of the molars was retarded. The changes in the incisor teeth in avitaminosis A have been described by Wolbach & Howe (1933), Pohto (1938), Irving & Richards (1939) and Schour *et al.* (1941). The chief action is on the odontoblasts which lay down a defective dentin, and excessively so on the labial side. Later, the enamel organ atrophies and the teeth lose their orange pigment. The effect of excess of the vitamin upon the alveolar bone has not been reported before. Pohto (1938) found that hypervitaminosis A had no effect upon the incisor teeth. Wolbach (1946) stated that the 'sequences of enamel and dentin formation are unaffected, perhaps they are somewhat accelerated'.

METHODS

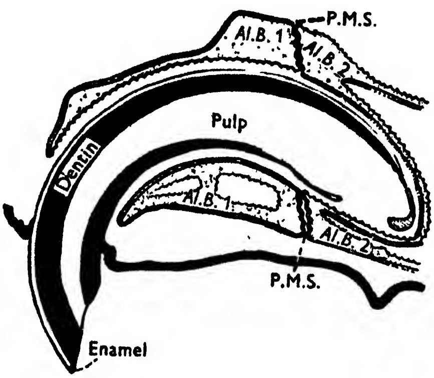
Hypervitaminosis A. Through the kindness of Dr T. Moore, and his associates, the skulls of fourteen hypervitaminotic rats were put at the disposal of the present writer. The experimental details, diet and treatment of the animals were essentially the same as described by Moore & Wang (1945). In brief young rats were given a basal diet similar to that used by these writers, with the inclusion of halibut liver oil in a proportion calculated to provide about 40,000 i.u. of vitamin A daily for from 10 to 42 days. Five control animals received a diet containing arachis oil, in place of halibut liver oil, but a supplement of halibut liver oil was given to each rat so as to supply about 150 i.u. of vitamin A daily.

The heads of the rats were skinned and the skulls were sent from Cambridge to Cape Town in 5% formol-saline. The lower jaws were removed, and the upper portions of the skulls were decalcified and embedded in paraffin. The incisor teeth were cut semi-serially in longitudinal section and stained with haematoxylin and eosin, and also by the method of silver impregnation (Gömöri, 1937).

Avitaminosis A. The tooth sections were from avitaminous rats previously investigated for a different purpose (Irving & Richards, 1939, 1940), and included the periodontal tissues. The diet employed and general management of the animals were the same as described by Irving & Richards (1938). Two different groups of specimens were examined. The first group was of animals on the vitamin A-free diet from weaning for periods of from 28 to 53 days. The second group was of animals on graded doses of the vitamin of from ½ to 4 i.u. daily for from 45 to 180 days from weaning. One rat had been on the vitamin A-free diet from 180 to 450 days of age. Each group had one or more control animals on the basal diet plus 80 i.u. of vitamin A daily. In all, 109 sections were examined.

RESULTS

Growth sequences in the alveolar bone of normal young rats. In order to understand the changes to be reported, a brief description must be given of the normal growth processes. These have been analysed in some detail by Sicher & Weinmann (1944) and by Weinmann & Schour (1945*b*). The alveolar bone consists of an anterior and posterior portion, divided by the premaxillo-maxillary suture, the anterior portion being thus part of the premaxillary bone, and the posterior portion of the maxilla (Text-fig. 1). The alveolar bone is different on the labial and lingual surfaces of the socket. The labial alveolar bone is divided into two almost equal parts by the premaxillo-maxillary suture, whilst this suture cuts through the lingual alveolar wall near the fundus. The fundic plate, a thin layer of bone opposite the basal end of the tooth, is part of the maxilla. On all these various parts of the alveolar bone, an internal, periodontal or alveolar surface, facing the tooth, has to be differentiated from the external surface, which faces marrow spaces, nasal cavity, nasolacrimal duct or forms the outer surface of the bone.



Text-fig. 1. Semi-diagrammatic drawing of the upper incisor of a normal rat. The alveolar bone consists of anterior (premaxillary, *Al. B.* 1) and posterior (maxillary, *Al. B.* 2) portions, which are separated by the premaxillo-maxillary suture (*P.M.S.*). Scalloped borders indicate sites of resorption; double bars with crossbars indicate sites of apposition. Reproduced with permission from Weinmann & Schour (1945*b*).

Owing to the normal growth and eruption of the tooth, a process of apposition and resorption is continually occurring in different parts of the alveolar bone. Resorption occurs in the premaxillary part of the periodontal face of the labial alveolar bone owing to growth in width of the incisor, while apposition takes place on the external side. Apposition occurs in the anterior part of the periodontal portion of the lingual alveolar bone and in the palatal plate, while resorption occurs in the marrow cavities inside the bone and on the bone surrounding the naso-lacrimal duct, which runs through the bone under the incisor tooth (Irving, Weinmann & Schour, 1947). Owing to its shift towards the tooth by sutural growth at the premaxillo-maxillary suture, the fundic bone is resorbed on the periodontal side, while apposition occurs on the external surface. The maxillary alveolar bone at the labial surface undergoes apposition on the periodontal face and resorption on the other side.

Hypervitaminosis A

Gross pathological findings (as reported by Dr Moore). The control animals were normal in all respects. All but two of the experimental animals had fractures of one or more bones and several had scattered haemorrhages. Three

died spontaneously. Both the blood and liver vitamin A values were considerably raised by the excessive dosage.

Alveolar bone. The most striking feature of the alveolar bone of the hypervitaminotic rats was its abnormal narrowness and fragile appearance as compared with that of the controls. The lingual alveolar bone, especially between the naso-lacrimal duct and the periodontal membrane, was extremely thin, and in some cases was missing in parts (Pl. 1, figs. 1 and 2). The labial alveolar crest was narrower than usual and in some cases seemed to have been split up into fragments.

Closer examination of the various parts of the alveolus indicated that while osteoclasis was proceeding, apposition of bone was in many places lacking. The lingual alveolar bone was bounded on the periodontal side by well-marked resting lines which extended from the alveolar crest to the premaxillo-maxillary suture. The spicules of the suture in the palatal plate were also bounded by cementing lines. The periodontal membrane on the lingual side of the tooth appeared normal.

The labial premaxillary alveolar bone showed osteoclastic resorption of normal intensity along its whole periodontal surface from the alveolar crest to the premaxillo-maxillary suture. Relatively much less apposition than usual appeared to be occurring on the external face. In some areas no apposition was occurring, while in others some osteoid-like material was being deposited. Many more cementing lines than usual were present in the whole labial alveolar bone, indicating that apposition had frequently stopped. The periodontal membrane was normal in all but one rat where it was compressed, probably by collapse of the alveolar bone.

The fundic bone was narrower than normal, in most cases stained more intensely with haematoxylin, and contained almost a mosaic of cementing lines (Pl. 1, figs. 3 and 4). Osteoclasts were present on the periodontal surface.

These changes in the alveolar bone seemed to be equally severe in all rats examined, irrespective of the length of time of hypervitaminosis. They were particularly marked in the rat which had died after 10 days, in which parts of the premaxillary labial alveolar bone were missing. In general, it could be concluded that the chief change was an imbalance of apposition and resorption, the first process being in large measure suppressed, so that the bone became unduly thin. This imbalance was most prominent in the premaxillary alveolar bone and in the fundic bone.

Incisor teeth. The changes in the dentin were best seen on the labial side of the tooth, and the following findings refer to investigations of this area. The time of transference of the rats to the basal diet at the commencement of the experiment was marked by a change in the histological appearance of the dentin laid down at that time. With haematoxylin and eosin, a faint hypocalcified layer followed by a hypercalcified zone was seen; with silver

impregnation a very clear line taking the silver stain less well was visible in all specimens (Pl. 1, figs. 5 and 6). The dentin produced during the experimental period was incrementally laid down in stripes staining alternately dark and light, in almost all teeth, both control and experimental, and the number of stripes when counted equalled the days of the experimental period, thus showing that this was in fact experimental dentin. Measurements of the width of this experimental dentin showed that in the control animals the average daily increment of dentin was 16 μ., which is the normal figure. In all the experimental animals the amount of dentin laid down during the experimental period was less than this, and the daily increment was on an average 13 μ. In several of the experimental animals the daily increments became narrower as one approached the pulpal side, indicating that the incremental growth of the dentin had gradually slowed down.

The predentin of the teeth of the experimental animal was narrower than normal, especially at the formative end of the tooth. The average predentin width at the formative end of the tooth was 18 μ. in the control and 9 μ. in the experimental animals. The figures obtained were about the same in all animals examined, irrespective of the length of time of hypervitaminosis. The fact that the experimental predentin width was less than the average daily increment is probably due to the gradual slowing of the incremental dentin formation. The narrow predentin was not the result of an imbalance between calcification and incremental growth. Had this been so, the predentin would have been still narrower, or even non-existent in the animals on the experiment for a long time, which was not the case.

The experimental dentin appeared more compact and stained somewhat more deeply with haematoxylin than the pre-experimental dentin, or than that of the controls. With the silver stain, the experimental dentin first laid down stained less deeply than the pre-experimental dentin, but that most recently formed stained more deeply and contained well-marked strands of fibrils running parallel with the pulpal border (Pl. 1, fig. 6). These were identified as the fibrils normally present in dentin, which were present in higher concentration per unit volume in this area, and which normally take the silver stain; the interfibrillar-cementing substance, which is not stained with silver, was relatively reduced in amount, indicating a decrease in mineral content. This change in the structure of the dentin appeared to coincide with the reduction in daily apposition rate.

The odontoblasts on the labial side of the pulp of the experimental animals were normal in appearance, but those on the lingual side at the formative end were reduced in height and at the epithelial sheath consisted of a line of flattened cells. The average height of these cells in the control animals varied from 25 to 64 μ., while in the experimental animals the corresponding figures were 17–46 μ.

The organic enamel was normal in all the experimental animals. Cysts were present in the enamel organ in two experimental animals. These have also been described by Weinmann & Schour (1945*a*) in rachitic animals and are probably not specific to hypervitaminosis A. The cementum was normal in all cases. In general it could be stated that the changes in the teeth in hypervitaminosis A were much less in degree than those in the alveolar bones.

Avitaminosis A

Alveolar bones. The changes to be described were already visible after 28 days on the vitamin A-deficient diet and became gradually greater with time. The protection afforded by various doses of the vitamin became less as the animals grew older, suggesting that the requirements rise with age (Irving & Richards, 1939).

The changes in the alveolar bones were essentially the same in all animals up to 180 days on the deficient diet, differing only in degree. In general, the chief change was an excess of apposition of bone compared with the opposite process of osteoclasis. Apposition occurred in some situations where normally resorption takes place. This was particularly noticeable at the labial alveolar crest and along the premaxillary labial bone. Here a reversal of the normal process was seen. In the early stages of the deficiency, osteoblasts could be sometimes seen 'elbowing out' osteoclasts on the periodontal surface. By 52 days on the deficient diet, only osteoblasts were seen (Pl. 1, fig. 7) and a wide layer of new bone, apparently normally calcified, had been laid down. On the external side of this bone osteoclasts were found, but here the normally occurring osteoblasts were also seen, and apposition and resorption might occur in adjacent areas.

The maxillary labial bone was almost normal in width in the early stages of the deficiency, but became much wider with marked apposition of new spongy bone on its periodontal surface in later stages. Osteoclasts were present on the opposite face in about normal numbers.

The fundic bone, in later stages of the deficiency, showed apposition on its periodontal face, instead of the usual resorption, and active osteoblasts could be seen (Pl. 1, fig. 8; cf. fig. 3). The new bone was not laid down as a regular layer, but as tongue-shaped outgrowths into the periodontal tissues. It was not uncommon in the early stages to find osteoclasts apparently endeavouring to remove this recently formed new bone and lying in close proximity to osteoblasts. Osteoclasts were sometimes seen in small numbers on the opposite face of the bone, but were not at all conspicuous.

Thus the chief change observed was an excess of osteoblastic activity. In sites where this occurred normally it went on unchecked and osteoclasts never appeared. While in areas where this action was abnormal, osteoclasts attempted in the early stages to remove this new bone being laid down. In

spite of this excessive apposition, bone growth as a whole was retarded (Schour *et al.* 1941).

The one rat examined which had been on the diet from 180 to 450 days of age showed changes somewhat different from those in the younger animals. Active apposition and resorption had largely stopped, presumably owing to cessation of bodily growth, since the same was found in the control animal. The alveolar bone throughout was much thicker and almost unrecognizable in places, consisting of cancellous and compact bone. This was true of the premaxillary alveolar bone on both sides and especially of the maxillary labial and fundic bones which, instead of being thin plates, were replaced by thick aggregates of bone.

Incisor teeth. The changes in these teeth in avitaminosis A have already been described by Wolbach & Howe (1933), Pohto (1938), Irving & Richards (1939) and Schour *et al.* (1941). The same changes were seen in the teeth of the present animals. On the whole, the teeth appeared less sensitive to this deficiency than the alveolar bones, since the teeth of animals killed early in the experiment were normal while the bones were already beginning to show the characteristic changes.

DISCUSSION

Wolbach's (1946) thesis, that resorption ceases in avitaminosis, while apposition continues is not supported by the above findings. Osteoclastic activity was changed and overshadowed by the excessive activity of the osteoblasts, but in no cases had it actually ceased. In addition, his statement that apposition occurs 'in strict conformity to normal growth patterns, both as to situation and rate', is not borne out by the present work, either with regard to situation or to rate. Nor was his concept of hypervitaminosis causing an acceleration of bone remodelling supported by the findings in the alveolar bones, since the present writer found that in such a condition the bones were slowly resorbed.

The changes in the alveolar bones were much more like those described by Mellanby, though here too some differences were discernible. The overactivity of the osteoblasts in normal and abnormal situations was just as described by him. He emphasizes the orderliness of the change in position of the osteoblasts and osteoclasts. His experimental animals were on the deficient diet for a considerable time, showed well-marked signs of deficiency, and the change-over of situation of the osteoblasts and osteoclasts was complete. It is difficult to picture, however, that at a certain time during the deficiency, these cells suddenly change places. The present results show that in the early stages this is not an orderly proceeding, but one in which the osteoclasts compete for their normal position with osteoblasts and are finally ousted. Even on the other surface of the bone, they are found between osteoblasts and their efforts are

quite insufficient to counteract the excessive apposition. Thus in the ultimate stages an appearance of orderly disorder appears to exist, but actually it is due to the preponderance of one type of cell over the other. Mellanby (1947) has advanced some attractive hypotheses to account for the apparent transfer of these cells from one part of the bone to another, but at present very little positive evidence exists to explain this phenomenon.

Mellanby has not reported the effects of hypervitaminosis, but he has investigated the effects of vitamin A administration upon vitamin-deficient animals. From the drawings in his paper (1947) it would appear that the osteoclasts respond with much greater vigour to this treatment than do the osteoblasts. In the present results, the osteoclasts were hardly affected by the hypervitaminosis. The chief feature noticed was the cessation of action of the osteoblasts.

Schour *et al.* (1941) have shown that the effect of vitamin A on the incisor tooth is specifically upon histodifferentiation of the odontogenic epithelium. It is thus of interest that the odontoblasts respond in a way very similar to that of the osteoblasts, and suggests that the underlying processes of differentiation of bone and tooth cells may be comparable. In vitamin A deficiency, the odontoblasts lay down an excessive amount of poorly formed dentin on the labial side of the tooth and may also produce osteoid masses in the pulp. In hypervitaminosis, the odontoblastic activity is damped down, and dentin formation becomes slower. The changes found in the present studies were quite different from those of Wolbach (1946), who stated that, if anything, enamel and dentin formation were accelerated. The quantitative deposition of fibrils does not seem to be reduced, and, as apposition slows, they become more concentrated and are thus more conspicuous, and there is a relative reduction in the amount of interfibrillar cementing substance and thus also in the Ca content per unit volume. If this also occurs in bone in hypervitaminosis, it may account for the increased fragility and tendency to fractures.

From the results reported in this paper it seems legitimate to postulate that the action of vitamin A in bone formation is primarily on the osteoblasts, these cells acting in an uncontrolled way in the absence of the vitamin, and being suppressed in hypervitaminosis. The reactions of the osteoclasts are purely secondary to this: in the former condition their action is quite obscured by the excessive bone production with which they are unable to cope; while in the latter, the osteoclasts work unopposed, removing and weakening bone, and finally causing fractures.

In the case of the teeth, the action of the vitamin is on comparable cells to the osteoblasts, namely the odontoblasts. The reactions of these cells are similar to those of the osteoblasts in the two conditions of vitamin A administration. The absence of cells analogous to osteoclasts makes the picture of the dental changes a much simpler one than that in the bones.

SUMMARY

1. The reactions of the alveolar bone and upper incisor teeth in hypervitaminosis and avitaminosis A have been studied in young rats.

2. In hypervitaminosis the rate of formation of bone is greatly reduced and active osteoblasts become much less prominent. Osteoclasts appear to be unaffected, with the result that the bones become abnormally thin and may disappear in places.

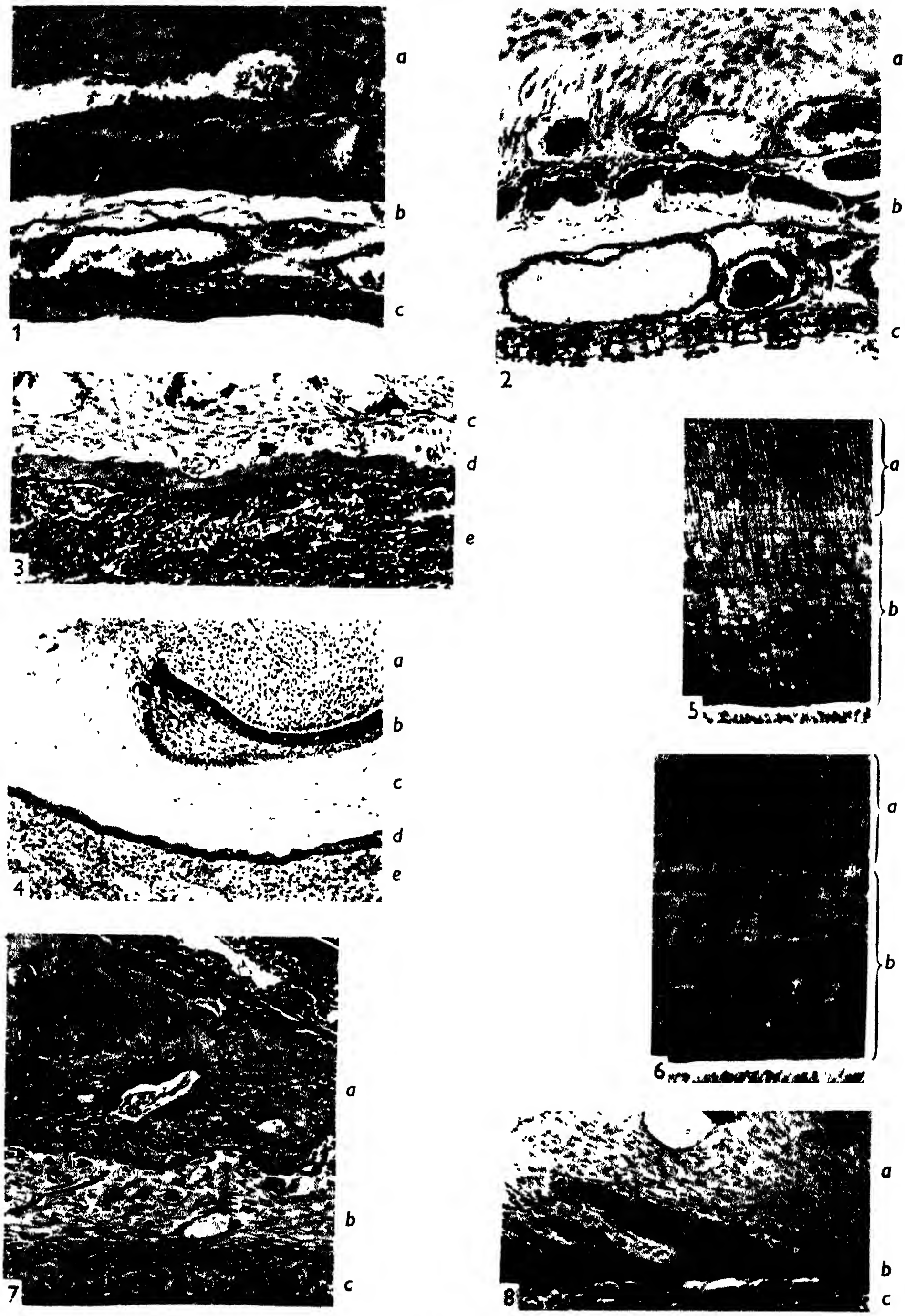
In the incisor teeth, only dentin formation is affected. This becomes decreased in appositional rate, the interfibrillar cementing substance is gradually reduced in amount, and the lingual odontoblasts begin to atrophy.

3. In avitaminosis, the alveolar bones show, in general, considerable overproduction of new bone. This occurs in areas where apposition is usually seen, and also in situations where resorption normally occurs In the early stages of the deficiency osteoclasts endeavour to overcome the latter unnatural apposition.

The teeth show changes already reported by other workers. These are chiefly in the dentin and odontoblasts, and excessive and faulty dentin formation occurs especially on the labial side.

4. The proposition is advanced that, in bone formation, vitamin A acts primarily on the osteoblasts. Excessive vitamin A in the diet depresses the action of these cells, while, when the vitamin is lacking, these cells engage in disorderly overactivity. The reactions of the osteoclasts in avitaminosis are purely secondary in an attempt to prevent excessive bone formation. In hypervitaminosis these cells continue to act as usual. The odontoblasts, cells in the teeth comparable to the osteoblasts, react in a way similar to that of the bone-forming cells, producing less dentin in hypervitaminosis, and excessive amounts in avitaminosis.

The writer is greatly indebted to Dr T. Moore and his colleagues, Dunn Nutritional Laboratory, University of Cambridge, and especially to Mrs S. E. Walker, for access to the hypervitaminotic material described in the present paper. This paper was read in manuscript by Drs I. Schour and J. P. Weinmann, University of Illinois College of Dentistry, and by Dr H. Sicher, Loyola University School of Dentistry, Chicago; the writer gratefully acknowledges their comments. The expenses of this work were met by grants from the Staff Research Fund, University of Cape Town, and from the South African Council for Scientific and Industrial Research; for these grateful acknowledgement is made.



Figs. 1–8.

To face p. 100

REFERENCES

Gömöri, G. (1937). *Amer. J. Path.* **13**, 993.

Irving, J. T. & Richards, M. B. (1938). *J. Physiol.* **94**, 307.

Irving, J. T. & Richards, M. B. (1939). *Nature, Lond.*, **144**, 908.

Irving, J. T. & Richards, M. B. (1940). Unpublished results.

Irving, J. T., Weinmann, J. P. & Schour, I. (1947). Unpublished results.

Mellanby, E. (1938). *J. Physiol.* **94**, 380.

Mellanby, E. (1944). *Proc. Roy. Soc.* B, **132**, 28.

Mellanby, E. (1947). *J. Physiol.* **105**, 382.

Moore, T. & Wang, Y. L. (1943). *Biochem. J.* **37**, *P* viii.

Moore, T. & Wang, Y. L. (1945). *Biochem. J.* **39**, 222.

Pohto, M. (1938). *Mikroskopische Untersuchungen über die Schneidezähne der Ratten bei der A-avitaminose, der Heilung derselben und der A-hypervitaminose.* Helsinki: The University.

Schour, I., Hoffman, M. M. & Smith, M. C. (1941). *Amer. J. Path.* **17**, 529.

Sicher, H. & Weinmann, J. P. (1944). *Amer. J. Orthodontics & Oral Surg.* **30**, 109.

van Metre, T. E. (1947). *Johns Hopk. Hosp. Bull.* **81**, 305.

Weinmann, J. P. & Schour, I. (1945*a*). *Amer. J. Path.* **21**, 821.

Weinmann, J. P. & Schour, I. (1945*b*). *Amer. J. Path.* **21**, 833.

Wolbach, S. B. (1946). *Proc. Inst. Med. Chicago.* **16**, 118.

Wolbach, S. B. & Bessey, O. A. (1941). *Arch. Path.* **32**, 689.

Wolbach, S. B. & Howe, P. R. (1933). *Amer. J. Path.* **9**, 275.

EXPLANATION OF PLATE

The sections are all of the upper incisor teeth or of the alveolar bone surrounding them. Magnification × 106.

Figs. 1 and 2. Lingual alveolar bone in the neighbourhood of the naso-lacrimal duct. *a*, periodontal membrane; *b*, lingual alveolar bone; *c*, epithelium lining the naso-lacrimal duct. Fig. 1 from control rat 1245, Fig. 2 from rat 1239, which died after 10 days' hypervitaminosis A. Note the thinning and fragmentation of the bone in Fig. 2.

Figs. 3 and 4. The fundic bone and adjacent tissues. *a*, pulp; *b*, odontogenic organ; *c*, periodontal membrane; *d*, fundic bone; *e*, mucous gland. Fig. 3 from control rat 4243. Fig. 4 from rat 1238 after 13 days' hypervitaminosis A. Note the very narrow fundic bone in Fig. 4, and the many cement lines.

Figs. 5 and 6. Labial dentin of the upper incisor tooth stained by Gömöri's method of silver impregnation (1937). *a*, pre-experimental dentin; *b*, experimental dentin, separated by a zone taking the silver less well. Fig. 5 from control rat 1244. Fig. 6 from rat 1242 after 13 days' hypervitaminosis A. Note the prominent fibrils in Fig. 6, especially in the most recently formed dentin.

Fig. 7. Labial alveolar bone of rat 4213 after 54 days' avitaminosis A. *a*, alveolar bone; *b*, periodontal membrane; *c*, enamel organ. Note the wide layer of new bone, bordered with osteoblasts, laid down on the periodontal side of the alveolar bone, separated from the old bone by a reversal line.

Fig. 8. Fundic bone region, from rat 3617, which received ¼ i.u. vitamin A for 50 days. *a*, periodontal membrane; *b*, fundic bone; *c*, mucous gland. Note the overgrowth of fundic bone into the periodontal membrane. The original size of the fundic bone can be seen outlined by a cement line (cf. Fig. 3).

J. Physiol. (1949) 108, 102–103 612.085.14

AN APPARATUS FOR RECORDING THE OUTPUT AND CORONARY FLOW IN THE HEART-LUNG PREPARATION

By R. P. STEPHENSON

From the Department of Pharmacology, University of Oxford

(*Received* 16 *February* 1948)

The apparatus recently described (Stephenson, 1948) for recording the outflow from small perfusions has been adapted and used to record the much larger flows which occur with the heart-lung preparation of the dog. The apparatus is shown in Fig. 1. The blood from the heart enters the apparatus through the glass tube (*a*) which reaches to the bottom of the wider tube (*b*). The flow from this tube is restricted by a third tube (*c*), and the amount of blood in (*b*) adjusts itself until the pressure it exerts is sufficient to send as much blood out at (*c*) as is entering at (*a*). Any alteration in the rate of flow produces a corresponding adjustment of the level of fluid in (*b*), and the change in volume is recorded on smoked paper with a piston recorder. The lower end of the tube (*a*) is turned up to ensure that the blood is continually mixed and does not stagnate in the upper part of tube (*b*). The sensitivity of the apparatus can be altered by changing the tube (*c*). The narrower the tube the more sensitive the apparatus.

An example of the use of two such recorders is given in Fig. 2. The upper record is of the outflow from the coronary sinus collected from a Morawitz cannula. The middle record is of the systemic outflow from the brachio-cephalic artery. At the beginning of the record the coronary outflow was 44 c.c./min. and the systemic output was 790 c.c./min. When the arterial resistance was lowered from 110 to 90 mm. Hg, the coronary flow fell from 44 to 36 c.c./min. With the reduction in resistance there was a transient rise in the systemic output from 790 to 840 c.c./min. After about 8 min. the arterial resistance was raised to 105 mm. and the coronary flow in consequence rose to 43 c.c./min. With the rise of resistance there was a transient reduction in systemic output to 740 c.c./min.

The continuous records of flow make it possible to demonstrate sudden and transient changes, and to show the dependence of coronary flow on arterial pressure in a simple manner.

The blood leaving the tube (*c*) can be collected in a measuring cylinder and the record thus calibrated. This should be done repeatedly in the course of each experiment, as the sensitiveness of the recorder depends on the height of the column of blood in (*b*).

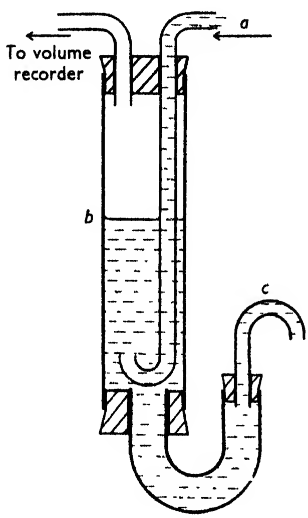


Fig. 1.

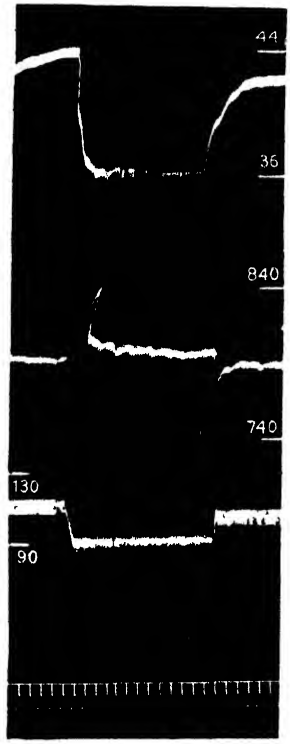


Fig. 2.

Fig. 1 Diagram of outflow recorder (see text).

Fig. 2. Heart-lung preparation. Upper record, outflow from coronary sinus (c.c./min.). Middle record, systemic outflow (c.c./min.). Lower record, arterial resistance (mm. Hg). Time 30 sec

The dimensions of the recorders used in the experiment shown in Fig. 2 were as follows: for the output of the heart, tube (*b*) was 2·15 cm. internal diameter, tube (*c*) was 0·43 cm. internal diameter and about 7 cm. long, and the piston recorder had an internal diameter of 3·2 cm.; for the record of coronary flow the corresponding dimensions were 1·70, 0·22 and about 5 cm., with a piston recorder of 1·8 cm.

I am indebted to Dr Edith Bülbring for providing me with Fig. 2.

REFERENCE

Stephenson, R. P. (1948). *J. Physiol.* **107**, 162.

J. Physiol. (1949) 108, 104–115 547.435.292

THE RELATION BETWEEN THE MOTOR AND INHIBITOR ACTIONS OF ACETYLCHOLINE

By J. H. BURN and J. R. VANE

From the Department of Pharmacology, University of Oxford

(*Received* 22 *March* 1948)

Recently different workers have recorded the stimulant action of acetylcholine in cardiac tissue. Sawaya (1939), working on the heart of the crustacean *Callinectes danae*, observed that acetylcholine increased the heart rate, and that acetylcholine is present in the heart of this species. Hoffmann, Hoffmann, Middleton & Talesnik (1945) have shown that the isolated cat heart, after treatment with atropine, responds to acetylcholine in a manner similar to that in which it responds to adrenaline. They observed that the increased contractions were accompanied by the appearance of an adrenaline-like substance in the perfusate. The perfusate relaxed the isolated intestine and the hen's rectal caecum, while it stimulated the frog heart. This observation has been confirmed by McNamara, Krop & McKay (1948). Hoffmann *et al.* found that nicotine abolished the stimulating action of acetylcholine; they concluded that acetylcholine acted on either ganglia or chromaffin tissue liberating an adrenaline-like substance which was responsible for the acceleration. McDowall (1946) also observed that acetylcholine stimulated the cat heart after atropine, and recorded a brief stimulant action of very small doses of acetylcholine in the heart not treated with atropine. In cat hearts in which the A.V. bundle was not divided, acetylcholine caused increased contraction of the ventricle. McDowall explained the stimulating action of acetylcholine as being in the main an effect on the ventricle in which it increased the force of contraction.

Observations of a reversed action of the vagus on the heart have been made by several observers. In 1910 Dale, Laidlaw & Symons described an increase of heart rate in the cat caused by vagal stimulation after the administration of tropine, nicotine, hordenine methiodide or curare. They observed that when the stimulation stopped, the rate dropped, giving the impression that the usual inhibitory effect was delayed. They were uncertain whether the phenomenon was due to a reversal of the function of fibres normally inhibitory in effect, or to the presence in the vagus of accelerator fibres which normally were masked.

In the course of examining the pharmacological action of the antimalarial substance known as paludrine (N′-*p*-chlorophenyl-N[5]-*iso*propyl biguanide), its effect on the heart was determined. Under its influence cardiac tissue was found to be so modified that doses of acetylcholine, which previously produced inhibition, produced stimulation. The circumstances of this change led to observations on smooth muscle also, which indicate a relation between the motor and inhibitor effects which acetylcholine produces.

Results

Isolated rabbit auricles. The auricles of the rabbit heart when dissected free from other tissue contract spontaneously for several hours in well-oxygenated Ringer-Locke solution. They maintain activity better at 29° than at 37° C.

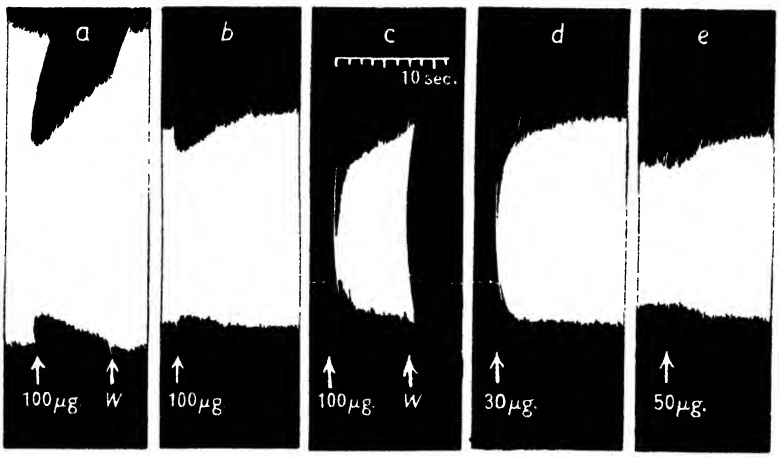


Fig. 1. (*a*) Spontaneous contractions of rabbit auricle, showing inhibitory effect of adding 100 µg. acetylcholine to bath (50 ml.). At *W*, fluid changed in bath. (*b*) Record taken 20 min. after addition of 4 mg. paludrine to bath; inhibitory action of 100 µg. acetylcholine was greatly reduced. (*c*) Exposure to paludrine for 65 min. has stopped the contractions; they are restarted by addition of 100 µg. acetylcholine, and stop again when this is removed. (*d*) The contractions are restarted by 30 µg. acetylcholine; this was left to act for 10 min. before it was removed. (*e*) The contractions continued, and were augmented by 50 µg. acetylcholine.

When the contractions were recorded as in Fig. 1 *a*, the effect of adding acetylcholine to the bath was to reduce the amplitude and the rate (in this experiment from 92 to 72 per min.). The amplitude slowly returned towards its original height, which was fully regained when the bath was washed out with fresh Ringer-Locke solution. Paludrine was then added to the bath (4 mg. in 50 ml.), with the result that the amplitude and frequency of the contractions slowly declined, and after 20 min. became as shown in Fig. 1 *b*. Addition of acetylcholine at this point had much less inhibitory action, and indeed the inhibition gave place to

a small increase in amplitude. There was no change of rate. The bath was emptied and refilled with Ringer-Locke solution containing the same amount of paludrine, and the decline in amplitude continued until the auricles stopped 65 min. after first being exposed to it. The paludrine was then removed and the bath refilled with Ringer-Locke solution. The auricles remained still during the next 40 min. until acetylcholine was added to the bath in a dose of 100 μg., the resulting concentration being 2×10^{-6}, when the auricles began to beat again after a latent period of 90 sec. (see Fig. 1*c*). The beat continued while the acetylcholine remained in the bath, but it ceased promptly when the solution in the bath was changed to fresh Ringer-Locke. The beat began a second time when 30 μg. acetylcholine was added (Fig. 1*d*), and the auricles were then left in this solution for 10 min. On washing out after this longer period the beat persisted, though with smaller amplitude than in the presence of acetylcholine. Addition of 50 μg. acetylcholine (Fig. 1*e*) augmented th amplitude, but did not alter the rate.

The arrest of the natural contractions by paludrine was usually abrupt, as shown in Fig. 2*c*, though sometimes it was gradual, the amplitude steadily diminishing to zero. When the spontaneous contractions were restarted by the addition of acetylcholine, the first contraction was usually large; this can be seen in Fig. 1*c*. The subsequent contractions then started with a small amplitude and steadily increased.

Transition from inhibition to stimulation by acetylcholine. The experiment described shows that under the influence of paludrine the spontaneous contractions of the auricles slowly decline until they cease altogether, and that the auricles are then in a condition in which the effect of acetylcholine is to stimulate them. Acetylcholine will cause resumption of contractions, and, when the contractions are resumed, will augment their amplitude. It is important to follow the gradual change in the response to acetylcholine. When paludrine first affects the auricles, the inhibition produced by a given dose of acetylcholine becomes smaller; a point is then reached where there is a transient inhibition followed by augmentation, as illustrated in Fig. 2*a* and *b*. The augmentation became the dominant feature of the effect, and preliminary inhibition was seen only when a large concentration of acetylcholine was applied. Thus there was no sudden change from inhibition to augmentation; at first both small doses and large produced inhibition proportional to the size of the dose; the inhibitory effects then became smaller and preceded a phase of augmentation; later still the smaller doses produced simple augmentation, and the larger doses transient inhibition followed by augmentation. When, under the continued influence of paludrine, the auricles stopped altogether, acetylcholine restarted the contractions.

Transition from stimulation to inhibition. The reverse change from a stimulating to an inhibiting effect can be observed in preparations in which the

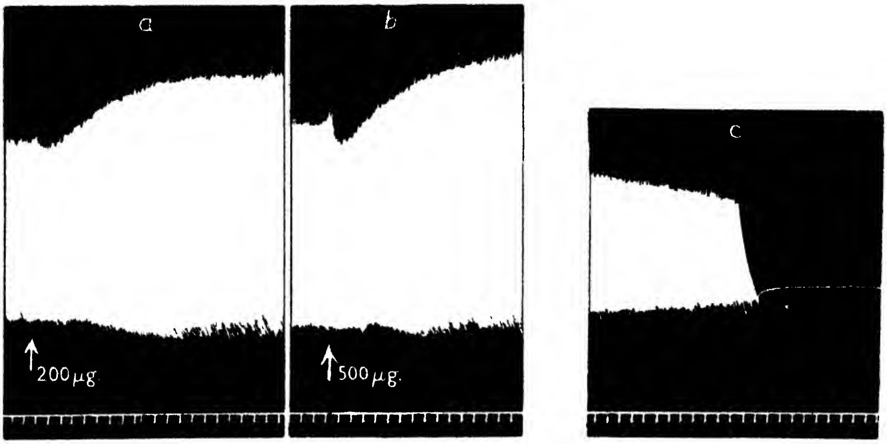


Fig. 2. (*a*) Auricles were exposed to paludrine; the spontaneous contractions diminished until at the point shown 200 μg. acetylcholine caused the rate to drop from 64 to 60 per min., but the amplitude to increase. (*b*) The addition of 500 μg. acetylcholine caused an initial diminution followed by augmentation of amplitude without change of rate. (*c*) The abrupt arrest of the auricles by paludrine. The rate fell from 44 to 40 to 36 per min. before the stop. Time 10 sec.

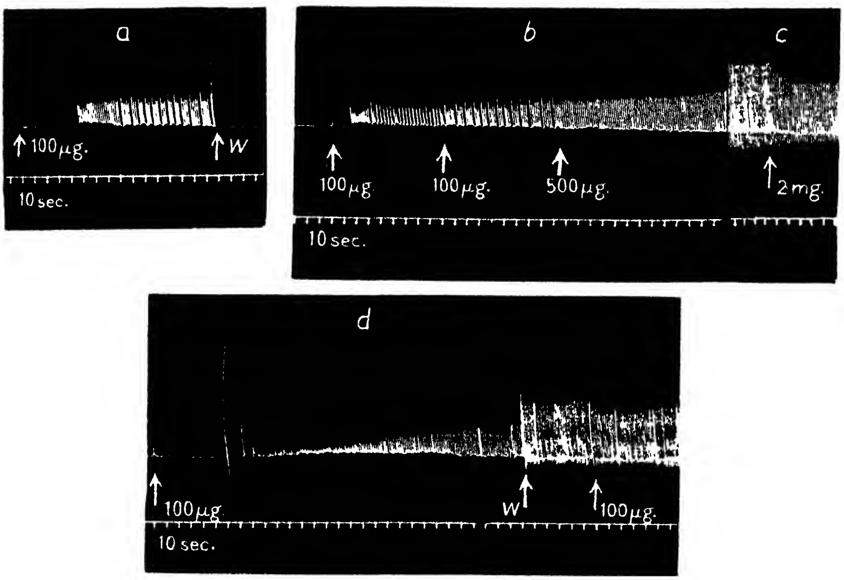


Fig. 3. (*a*) Auricles were arrested by exposure to paludrine. After removal of paludrine they were restarted by addition of 100 μg. acetylcholine. They stopped when it was washed out. (*b*) Auricles restarted by 100 μg. acetylcholine; the rate and amplitude were increased by a further addition of 100 μg. acetylcholine, and still more by 500 μg. acetylcholine. (*c*) When the amplitude increased further, the addition of 2 mg. acetylcholine caused diminution and slowing. (*d*) In another experiment the auricles were restarted by 100 μg. acetylcholine; this was removed by washing out at *W*. The beat continued for 12 min., and then a further addition of 100 μg. caused inhibition.

spontaneous contractions have been first stopped by paludrine. For example Fig. 3*a* shows a resumption of contractions when 100 μg. acetylcholine was added to the bath after the auricles had been still for 1 hr. When the acetylcholine was washed out, the contractions stopped again. Fig. 3*b* shows further resumption of contractions when acetylcholine was added and a steady increase in their rate, regularity and amplitude, as the concentration of acetylcholine in the bath was increased. After 10 min. the bath was washed out and the auricles then continued to beat as shown in Fig. 3*c*. At this point, addition of 2 mg. acetylcholine caused the ordinary inhibitory response. Thus in this experiment smaller doses restored spontaneous contractions and increased them, but when they were established a larger dose caused inhibition.

In another similar experiment (Fig. 3*d*) the change from a stimulant to an inhibiting action was observed without increase of dose. The quiescent auricles began to beat when 100 μg. acetylcholine was added to the bath. The beat steadily increased in amplitude, and after 12 min., when the bath was changed, the auricles continued to beat at the rate of 52 per min. When 100 μg. acetylcholine was added, the effect was to diminish the amplitude.

Removal of paludrine. During the observations described, paludrine was not added to the bath after the auricles had once stopped. It was therefore evident that paludrine would diffuse out of the tissue so that the contractions might resume without the addition of acetylcholine. This was tested by washing out the bath several times after the auricles had stopped; it was found that the auricles did in fact resume contractions. A series of observations was therefore made in which, from the moment the auricles ceased to beat, the bath was washed out at 10 min. intervals. The time until the auricles began to beat again was measured. This series was compared with another series in which acetylcholine was added to the bath 7 min. after each wash-out and left in contact

TABLE 1. Time elapsing until contractions resumed

	Controls	100 μg. ACh added for 3 min.	250 μg. ACh added for 3 min.
	41	20	20
	54	34	37
	70	49	43
	73	55	46
	76	58	47
	80	60	59
	81	—	—
	200	—	—
Mean	84	46	42

for 3 min. The results are given in Table 1. The number of additions of acetylcholine necessary to restart the contractions varied from 2 to 6, and all 12 auricles to which these additions were made started within 60 min. When no acetylcholine was added so that the resumption of contractions was due to removal of paludrine, only 2 of the 8 auricles began to contract in this time.

Even when the high figure of 200 min. in the first column was excluded (though there was no reason for excluding it) the significance of the difference between the means in the first and third columns was 3·3. That acetylcholine was responsible for restarting the auricular contractions was in any case proved by the observations in which the contractions stopped when acetylcholine was washed out.

Electrical stimulation. When the auricles were arranged so that they could be stimulated electrically (Dawes, 1946), and when the contractions were arrested by the addition of paludrine to the bath, it was found that they contracted in response to stimulation, but the amplitude was small, being equal to the amplitude at the point when the spontaneous contractions ceased.

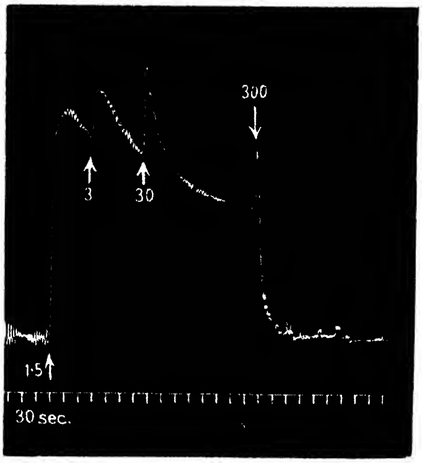


Fig. 4. Rabbit jejunum. Tyrode solution. Changes due to additions of acetylcholine to the bath, the figures being the amount in μg. per ml. of fluid in the bath present after each addition. 300 μg./ml. caused inhibition.

General properties of paludrine. The general properties of paludrine will be described by one of us (J.R.V.) elsewhere, but it may be remarked at this point that it depresses the action of acetylcholine in those tissues in which the action of acetylcholine is stimulant or motor. Thus paludrine depresses the stimulant action of acetylcholine on the isolated frog rectus and on the isolated guinea-pig intestine. Paludrine depresses the effect of vagal stimulation on the contractions of the cat's intestine. Thus a substance which depresses the stimulant action of acetylcholine in smooth muscle and in skeletal muscle was found to convert the inhibitory action of acetylcholine on the heart to a stimulant action by a gradual process. This suggested that a double effect of acetylcholine might be observed in other tissues, and that an inhibitory effect of acetylcholine in smooth muscle might be observed by increasing the concentration.

Observations on the intestine. Loops of rabbit intestine were isolated and suspended in a bath of Ringer-Locke, or of Tyrode solution aerated with oxygen + 5 % CO_2. The observations, which were made in about 20 loops from different rabbits, are illustrated in Fig. 4. The addition of acetylcholine (the bromide was the salt used) to make a concentration of 1·5 μg./ml. caused contraction of the loop. Further additions of acetylcholine to raise the concentration by 3, then by 30 and finally by 300 μg./ml. were then made as shown. After the final addition there was a momentary contraction followed by inhibition almost to the base line. Some sign of an inhibition was also seen after raising the concentration by 30 μg./ml.

Observations on the uterus. Experiments were performed using one horn of the non-pregnant uterus of the rat suspended in Tyrode solution aerated with oxygen and 5 % CO_2. An example of the results is shown in Fig. 5, in the first part of which acetylcholine added to produce a concentration of 12 μg./ml. caused a greater contraction than had occurred spontaneously before; the addition of a high concentration then caused inhibition. When the acetylcholine was removed, the uterus remained fairly quiescent, but (Fig. 5*b*) contracted when the concentration was raised to 12 μg./ml. As the contractions declined, the concentration was raised further by 100 μg./ml. and there were renewed contractions. The high concentration of 1000 μg./ml. then caused inhibition. After the acetylcholine was washed out, contractions were resumed. At this point, as shown in Fig. 5*c*, the addition of a concentration, namely 12 μg./ml., which previously caused contraction, now caused inhibition.

In other experiments it was observed that during the initial period of observation, the gradual raising of the concentration of acetylcholine in the bath even to 1000 μg./ml. did not cause inhibition. Thus additions were made of 25, 60, 250 and 1000 μg./ml. respectively. The first caused a sharp rise in tone and increased amplitude of rhythm; the succeeding additions caused a slight rise in tone, but there was no sign of inhibition. When the bath was washed out the uterus relaxed, and then resumed its rhythm. When now the concentration was raised to 125 μg./ml. followed after 8 min. by a further rise of 1000 μg./ml., the first caused stimulation, but the second caused an inhibition like that seen in Fig. 5*a*. The different effect of the high concentration of 1000 μg./ml. on the same muscle at the two times indicated in the first place that the inhibitory effect was not due to any change of tonicity or pH, and that it was facilitated by the previous exposure of the muscle to a high concentration of acetylcholine. This facilitation was repeatedly observed, as in Fig. 5*c*.

Observations on the aorta. The blood vessels are composed of tissue which is normally relaxed by acetylcholine. It is known that this is not the only response which can be obtained, for when the vessels of the rabbit ear are perfused with Ringer solution, after 24 hr. the injection of acetylcholine causes vasoconstriction. Observations were made on the aorta, spirals being cut from

the aorta of the freshly killed rabbit. These were suspended in a bath of Ringer-Locke solution and left for 24 hr. at room temperature, the lever being weighted

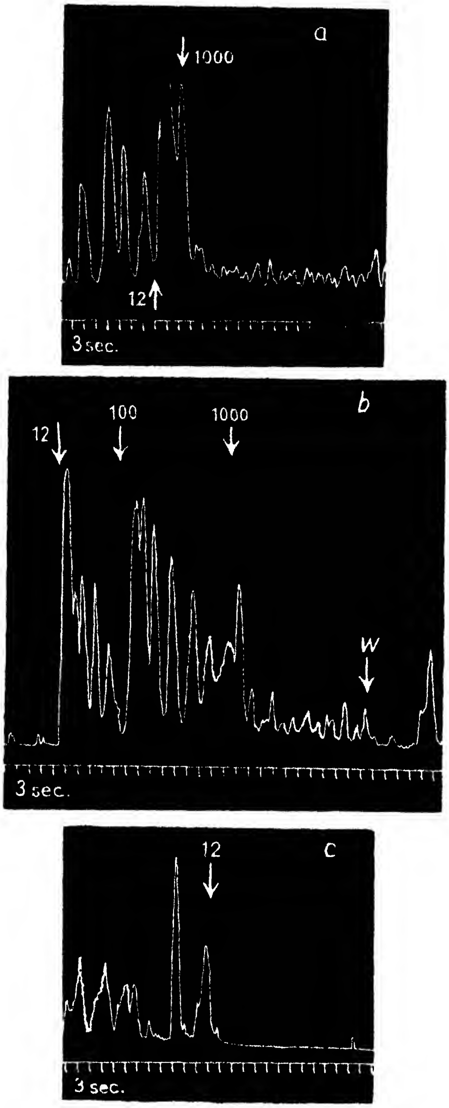


Fig. 5. Rat uterus. Tyrode solution. (*a*) Stimulation by addition of 12 μg./ml. acetylcholine. Inhibition by 1000 μg./ml. (*b*) Continuation of (*a*), showing stimulation by 12 and by 100 μg./ml. but inhibition by 1000 μg./ml. (*c*) Continuation of (*b*) showing inhibition by 12 μg./ml.

so as to stretch the spiral. The next day the bath was warmed to 37° and oxygenated. A concentration of 1 mg./ml. acetylcholine then regularly caused

contraction of the aorta. When the concentration in the bath was raised to 10 mg./ml., relaxation occurred, though it was not complete. The effect is illustrated in Fig. 6.

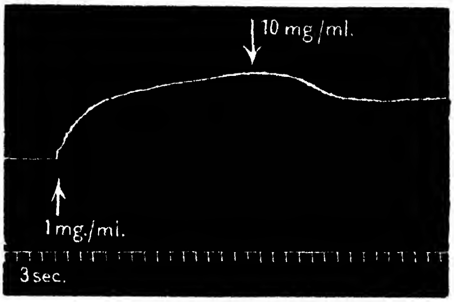


Fig. 6. Spiral cut from rabbit aorta, left in Ringer-Locke solution overnight until relaxed. Contraction caused by 1 mg./ml. acetylcholine; some relaxation by addition of 10 mg./ml.

Discussion

By exposing the isolated auricles of the rabbit to a constant concentration of paludrine we have been able to observe not only a change in the response to acetylcholine from inhibition to stimulation, but also that the change is gradual. It has occurred together with a gradual reduction in amplitude of the contractions, leading to complete arrest. When the auricles have thus stopped beating, we have observed that the addition of acetylcholine started the contractions once more, and that when the acetylcholine was removed, the contractions again stopped. At this point the presence of acetylcholine, so far from causing inhibition, initiated the automatic rhythm. In some experiments it was observed that each further addition of acetylcholine increased both the rate and the amplitude of the spontaneous contractions. Later, again when paludrine had diffused out of the auricular tissue, the normal inhibitory action returned, sometimes in response to a large dose, sometimes in response to a dose which shortly before had been used to restart the contractions.

Hitherto, in its relation to heart muscle, acetylcholine has been generally regarded as the transmitter of the inhibitory influence of the vagus nerve. Within the last few years, however, various observations have suggested that acetylcholine has another quite different function in the heart. In his search for substitutes for quinidine Dawes (1946) pointed out that substances which prolong the refractory period, such as procaine, quinidine and quinine are substances which depress the action of acetylcholine in many different tissues. They reduce its effect on the rate and amplitude of heart muscle and on the movements of the isolated intestine. Now the refractory period is the time required for the muscle to reload its contractile mechanism, and as has been shown by Dawes

(1946) and studied quantitatively by de Elio (1947), the addition of acetylcholine shortens this time. From these observations there emerged the suggestion that acetylcholine is not merely concerned in the humoral transmission of vagal impulses, but is present in heart muscle, playing an important part in the processes responsible for spontaneous contraction. The observation of Comline (1946) that the auricles contain the enzyme system which synthesizes acetylcholine in relatively large amounts conforms with this suggestion.

Though our acceptance of this as a hypothesis sprang from the work of Dawes, and gained support from that of others in this department (Dews & Graham, 1946; de Elio, 1947, 1948; Stephenson, 1948), the hypothesis is not new. It was put forward by Abdon (1945) as a result of observations on the presence of an acetylcholine precursor in the heart of the rabbit. Abdon spoke of 'the general appearance of tissue acetylcholine in many places where it could not have the function of a humoral transmitter', and said that 'the breakdown and formation of precursor belong to the normal metabolism of cardiac muscle'.

The immediate difficulty of the hypothesis was to explain why, if acetylcholine played a part in promoting the cardiac rhythm, the ordinary effect of adding acetylcholine was to arrest it. Paludrine shared the properties of substances like quinidine, procaine and some others, of prolonging the refractory period of rabbit auricles when tested by Dawes's method, and also of diminishing the response of other tissues such as the frog rectus, the rabbit intestine and the rabbit auricles to acetylcholine. Since the application of paludrine was found to lead to arrest of contractions which, however, could be restarted by the addition of acetylcholine, we supposed that paludrine must gradually depress the response of the tissue to acetylcholine as it depressed that of the isolated intestine, so that the amount normally synthesized became increasingly ineffective, and finally incapable of causing contraction to take place. Under these conditions, in which the threshold for acetylcholine is raised, the addition of acetylcholine to the bath might have the effect of increasing the concentration in the tissue to this threshold and thereby producing a contraction.

How then were we to regard the normal inhibitory action of acetylcholine on the heart, which, when the heart was depressed by paludrine, gradually became an augmentation? Since the process was reversed when paludrine was slowly removed, we conceived the idea that acetylcholine when externally applied might have two actions in an excitable tissue with rhythmic activity in which acetylcholine synthesis was proceeding. If the amount synthesized was less than the amount to which the muscular elements were capable of responding, as in the normal intestine, or in auricular tissue rendered insensitive to acetylcholine by paludrine, then the added acetylcholine would cause stimulation. On the other hand, inhibition would follow the addition of acetylcholine if the amount synthesized was the full amount to which the muscle elements were capable of responding, as in the normal auricles, or if the tissue was saturated

by previous additions. This view was tested by examining the effect of acetylcholine on the intestine, and on the uterus. In both of these tissues the enzyme system for acetylcholine synthesis is present (for the uterus, see Reynolds, 1939, and Emmens, MacIntosh & Richter, 1943). It was found that in preparations in which the effect of low concentrations of acetylcholine was stimulation, the addition of excess of acetylcholine caused inhibition.

In the intestine inhibition was produced by 250–300 μg./ml. acetylcholine bromide. In some preparations of the uterus, concentrations as high as 1 mg./ml. did not at first cause inhibition, though a second exposure to this concentration did so; in other preparations, after one or two exposures to a high concentration, it was sufficient to put in the bath 12 μg./ml. to observe inhibition.

A few experiments were made on the smooth muscle of the blood vessels. In the body acetylcholine causes dilatation, that is to say, inhibition. We found that if a spiral of rabbit aorta was allowed to relax when suspended in a bath, concentrations of 1 mg./ml. caused contraction, and ten times this concentration caused inhibition. Since further observations are being made on blood vessels, discussion of this admittedly slender evidence will be omitted, except to say that it too gives support to the hypothesis. The point we believe to be new in the present work is the suggestion that the double action, stimulation in low concentration, inhibition in high, is the key to the difference in the effect of vagus stimulation on the heart and on the intestine. It implies that in a tissue such as the heart (and perhaps the blood vessels) in which the normal action of acetylcholine is inhibitory, there exists a mechanism in which the production of acetylcholine is a normal feature and in which that production is at such a level that the addition of more acetylcholine causes inhibition.

SUMMARY

1. The action of acetylcholine on isolated rabbit auricles is gradually changed by exposure to paludrine from an inhibitory action to a stimulant action. This change proceeds *pari passu* with a diminution in the size of the spontaneous contractions.

2. When exposure to paludrine is continued, the auricles are arrested, and if paludrine is removed they remain still for long periods. Spontaneous contractions are restarted by exposure to acetylcholine. They stop once more when acetylcholine is removed.

3. When the auricles are restarted by acetylcholine, further additions augment the rate and amplitude of contractions. As paludrine diffuses out of the tissue, a point is reached at which the inhibitory action of acetylcholine is seen once more.

4. The action of paludrine is to be explained in the light of the fact that it depresses the response of other tissues such as the frog rectus and rabbit intestine to acetylcholine, and lengthens the refractory period of the auricles.

5. When the isolated rabbit intestine is exposed to high concentrations of acetylcholine, the tone and rhythm is inhibited. This is also true of the rat uterus.

6. A hypothesis is put forward to relate the stimulant and inhibitor actions of acetylcholine.

REFERENCES

Abdon, N. O. (1945). *Act. Pharmacol. Toxicol.* **1**, 169.
Comline, R. S. (1946). *J. Physiol.* **105**, 6*P*.
Dale, H. H., Laidlaw, P. P. & Symons, C. T. (1910). *J. Physiol.* **41**, 1.
Dawes, G. S. (1946). *Brit. J. Pharmacol.* **1**, 90.
Dews, P. B. & Graham, J. D. P. (1946). *Brit. J. Pharmacol.* **1**, 278.
de Elio, F. J. (1947). *Brit. J. Pharmacol.* **2**, 131.
de Elio, F. J. (1948). *Brit. J. Pharmacol.* **3**, 108.
Emmens, C. W., MacIntosh, F. C. & Richter, D. (1943). *J. Physiol.* **101**, 460.
Hoffman, F., Hoffman, E. J., Middleton, S. & Talesnik, J. (1945). *Amer. J. Physiol.* **144**, 189.
McDowall, R. J. S. (1946). *J. Physiol.* **104**, 392.
McNamara, B. P., Krop, S. & McKay, E. A. (1948). *J. Pharmacol.* **92**, 153.
Reynolds, S. R. M. (1939). *J. Physiol.* **95**, 258.
Sawaya (1939). *Bol. de Zool. Univ. S. Paulo.*
Stephenson, R. P. (1948). *Brit. J. Pharmacol.* **3**, 237.

J. Physiol. (1949) 108, 117–126 612.64.012

FRUCTOSE IN THE SHEEP FOETUS

By M. W. S. HITCHCOCK

From the Agricultural Research Council's Unit of Animal Physiology, University of Cambridge

(*Received* 5 *June* 1947)

The presence of fructose in the amniotic and allantoic fluids of a wide variety of mammals was reported by Paton, Watson & Kerr (1907). They also claimed its presence in the foetal blood of the sheep and cow. Orr (1924) showed that the filtrate from human foetal blood gave a Seliwanoff reaction. But until the work of Bacon & Bell (1946), in which fructose was isolated from foetal sheep's blood as a diacetone derivative, rigid proof as to the identity of the substance was lacking.

But while fructose had been reported to be present in the foetal blood of a number of species, nothing was known of the levels at which it or glucose were present throughout the course of pregnancy. Bacon & Bell (1946) had shown that glucose accounted for the whole of the difference between the total fermentable reducing sugars and fructose, and determinations which had been made of total sugar and fructose in the foetal and maternal bloods of sheep throughout the second half of pregnancy by Cole & Hitchcock (1946) were interpreted in this light. These last observations were, however, not reliable, since the blood-sugar level of the mother had on the average doubled in the period between giving the anaesthetic and taking the samples from the uterine and umbilical vessels. Consequently, it was felt to be most important that the observations be repeated on ewes in which the maternal blood-sugar level had been prevented from rising to such an extent.

While this was the main object to the present work the opportunity was also taken to determine the distribution of glucose and fructose between the foetal corpuscles and plasma.

METHODS

All the sheep used were Welsh mountain ewes with a gestation period of 147 ± 3 days.

Administration of anaesthetic and preparation of animal. The administration of the anaesthetic and the preparation of the animal presented much difficulty because of the tendency of the maternal blood-sugar level to rise when the animal was handled. Thus, while the normal blood

sugar of sheep approximates to 40 mg. % (Hitchcock & Phillipson, (1946), consistently higher levels were found in blood drawn from the uterine vessels of the maternal ewe by Cole & Hitchcock (1946). The question arose whether those high blood sugars were due to the experimental procedure adopted or to the tendency of the blood sugar to rise as the result of the administration of the anaesthetic. The effect of different anaesthetics was first investigated, the blood-sugar level in the maternal jugular being followed by taking a sample before administering the anaesthetic and at intervals during and after the usual dissection and experimental procedure. Urethane intravenously and duracaine (procaine hydrochloride) as a spinal anaesthetic were used in the two experiments, the results of which are given in Tables 1 and 2. It can be seen that a substantial rise took place in both cases.

TABLE 1. Sheep 717. Total sugar in blood from jugular vein; urethane given from 10.20 to 10.35 a.m.

Time (a.m. to p.m.)	9·45	10·45	11·15	11·45	12·15
Total sugar (mg. %)	57	68	83	82	83

TABLE 2. Sheep 719. Total sugar in blood from jugular vein; duracaine given at 10.40 a.m.

Time (a.m.)	10·35	10·55	11·10
Total sugar (mg. %)	38	53	54

The following was eventually found to be the most successful in keeping the blood sugar down to the level first observed. The sheep was brought into the laboratory 2 or 3 days before the operation and put into a pen. The wool was clipped off the neck over the jugular vein and off any other part which might ultimately be required to be free from it. On the day of the experiment, a hypodermic needle was inserted into the jugular vein and a sample of blood taken for analysis. An anaesthetic dose of nembutal (pentoburbital sodium) was immediately injected through the needle already in the vein and the sheep was then carried to the operating bath.

If the process of drawing the blood and giving the anaesthetic had been performed without any struggling on the part of the sheep, the total sugar was found to be within normal limits and to remain approximately constant during an operation lasting for upwards of an hour. It thus seemed clear that the high level of blood sugar found in the uterine vessels of earlier experiments was a function of the degree of agitation involved in the reaction of the sheep to manipulation. In all earlier experiments a spinal anaesthetic had been given, the sheep remaining conscious without suffering pain. On the other hand, a general anaesthetic would not in itself prevent a rise in blood sugar, unless its administration was accompanied by the precautions already described and the absence of struggling.

TABLE 3. Total sugar in blood from jugular vein and uterine vessels

	Sheep 729		Sheep 728		Sheep 725	
	Time (a.m.)	Total sugar (mg. %)	Time (a.m.)	Total sugar (mg. %)	Time (a.m.)	Total sugar (mg. %)
Jugular vein	9·58	38	10·01	33	10·50	33
Uterine vein	10·42	42	10·30	39	11·11	35
Uterine artery	10·44	42	10·32	42	11·13	37

TABLE 4. Sheep 623, 76 days pregnant. Sugars in blood from the uterine and umbilical vessels

	Total sugar (mg. %)	Fructose (mg. %)	Glucose (mg. %)
Uterine artery	110	1	109
Uterine vein	88	1	87
Loss by mother	22	0	22
Umbilical vein	149	103	46
Umbilical artery	143	100	43
Gain by foetus	6	3	3

The results given in Table 3 provide an idea of the degree of success which was attained. In Table 4 are given typical results of earlier experiments in which no precautions against a rise in the level of the maternal blood sugar were taken. The magnitude of the error introduced in the observations on the sugars in the blood from the uterine and umbilical vessels will be realized if they are compared with data given later in this paper. The results obtained by Cole & Hitchcock (1946) should therefore be regarded as unreliable to that extent.

Collection of blood. The majority of the determinations were of the levels of total sugar and fructose in blood drawn from the uterine and umbilical vessels. The samples were collected in the following manner: the abdominal cavity was opened and the samples from the uterine vein and artery taken with the least possible disturbance to the uterus. The uterus was then opened, the cord revealed and samples from the umbilical vein and artery taken. The blood was drawn by means of a hypodermic syringe, put into a beaker and shaken with 10–20 mg. sodium fluoride per c.c. of blood. An Ostwald pipette was used for measuring the required volume.

Blood from the foetal carotid was obtained through a cannula and was used for investigating the distribution of the sugars between corpuscles and plasma. Coagulation was prevented either by heparin or by a mixture of ammonium and potassium oxalates according to the method of Wintrobe (1942). Use was made of the haematocrit value for calculating the corpuscle sugars, determinations of total sugar and fructose being made on the whole blood and plasma only.

Chemical methods. Bacon & Bell (1946) have given rigorous proof that the blood of the sheep foetus contains only two sugars, fructose and glucose. Work by Karvonen (1948) makes it possible to go further and to state that the total reduction obtained with blood from the sheep foetus when the method of Somogyi (1945) is used for determining blood sugar, is referable to these two substances. Karvonen determined both total reducing substances (using Somogyi's method) and fructose in the foetal blood, before and after yeast fermentation. Table 5 gives examples of his results.

TABLE 5. Sugar determinations before and after yeast fermentations

	Before fermentation		After fermentation	
	Total sugar (mg. %)	Fructose (mg. %)	Total sugar (mg. %)	Fructose (mg. %)
Foetus H 7*a*	190	148	Nil	Nil
Foetus H 7*b*	165	116	Nil	Nil
100 mg. % Fructose	--	—	Nil	Nil

It was therefore possible to estimate:

(1) Total sugar by the method of Somogyi (1945).

(2) Fructose by Cole's modification of Roe's (1934) method as described by Bacon & Bell (1946).

(3) Glucose by the difference of (1) and (2).

The blood filtrates were prepared throughout by precipitating the proteins with cadmium sulphate and sodium hydroxide according to the method of Fujita & Iwatake (1931).

The following control determinations indicate the degree of accuracy obtained using the methods mentioned above. In Table 6 are shown results of determinations on sugar solutions, and in Table 7 those on a sample of normal sheep's blood. Hence the apparent 0·7 mg. % fructose in the blood of the adult sheep corresponds to a true fructose content of 0·4 mg. %.

Since the specificity of the method for fructose was so high, the correction only became significant when the fructose content was of the low order found in the blood of sheep and of lambs more than 1 day old; and as the estimation of such small quantities of fructose could not be considered accurate in any case, the fructose levels in the maternal blood are given to the nearest mg. % in the main results.

TABLE 6. Control determinations on sugar solutions

	10 mg % fructose	10 mg. % fructose and 5 mg. % glucose	
	Total sugar (mg. %)	Total sugar (mg. %)	Fructose (mg. %)
Determination 1	9·8	14·7	9·8
2	10·0	14·6	9·8
3	10·1	14·6	10·2
4	10·1	14·9	9·8
5	10·1		10·2
Average	10·0	14·7	10·0

TABLE 7. Control determinations on normal sheep's blood

	Total sugar (mg. %)	Fructose (mg. %)
Determination 1	47·1	0·7
2	47·1	0·7
3	47·8	0·8
4	47·8	0·6
5	47·1	
Average	47·4	0·7

Using blood from the same sample as that on which the above determinations were made a filtrate was prepared, containing a calculated fructose content of 9·6 mg. % and a calculated total sugar content of 17·8 mg. % by the addition of standard fructose and glucose solutions in the course of precipitation. The results of determinations on that filtrate are given in Table 8.

TABLE 8. Determinations on blood filtrate containing added fructose and glucose

	Total sugar (mg. %)	Fructose (mg. %)
Determination 1	17·4	9·60
2	17·2	9·60
3	17·4	9·52
4	17·2	9·76
5		9·68
Average	17·3	9·6
Percentage recovery	97	100

The method for determining fructose showed recoveries of 100 % from a mixture of fructose and glucose in aqueous solution and from blood; that for total sugar showed recoveries of 97–98 % for similar mixtures in aqueous solution and blood and 100 % for fructose alone in aqueous solution.

RESULTS

Levels of fructose and glucose in foetal and maternal bloods. Table 9 gives the values for total sugar, fructose and glucose respectively, found in the uterine and umbilical vessels during anaesthesia, and for the total blood-sugar level of the mother prior to the administration of the anaesthetic. In sheep no. 731, the latter had risen to 43 mg. % in the jugular vein at the time of taking the samples from the umbilical vessels.

Two trends can be observed from the table: a fall in the level of fructose in the umbilical vessels during the second half of the gestation period, and

TABLE 9. Total sugar, fructose and glucose in the blood of the uterine and umbilical vessels

Sheep no. ...	723	724	725	729	728	731	734	758	750
Foetal age (days) ...	64	74	85	99	100	106	116	132	145
Total sugar (mg. %.)									
Jugular Vein (maternal; before operation)	34	39	36	38	33	34	26	15	23
Uterine artery	46	46	37	42	42	—	37	17	22
Uterine vein	46	41	35	42	39	—	30	17	20
Umbilical vein	93	178	121	108	140	104	100	66	45
Umbilical artery	—	—	111	—	138	101	97	61	53
Fructose (mg. %)									
Uterine artery	1	2	1	1	1	—	1	1	1
Uterine vein	2	2	1	—	1	—	1	1	1
Umbilical vein	80	167	102	85	109	79	65	58	34
Umbilical artery	—	—	100	—	110	78	65	55	40
Difference	—	—	+2	—	−1	+1	0	+3	−6
Glucose (mg. %)									
Uterine artery	45	44	36	41	41	—	36	16	21
Uterine vein	44	39	34	—	38	—	29	16	19
Difference	+1	+5	+2	—	+3	—	+7	0	+2
Umbilical vein	13	11	19	23	31	25	35	8	11
Umbilical artery	—	—	11	—	28	23	32	6	13
Difference	—	—	+8	—	+3	+2	+3	+2	−2

a narrowing of the difference in the glucose levels of the maternal and foetal bloods. This second trend can be more clearly seen from Table 10, which shows the glucose level in the blood of the umbilical vein expressed as a percentage of that in the uterine artery. It will be noted that, in all the cases for which the trend holds, the glucose level in the blood of the uterine artery is normal for an adult sheep, but that in the two towards the end of pregnancy, at 132 and 145 days respectively, the glucose level in the blood of this vessel is definitely subnormal. This should not be taken as evidence that the maternal blood-sugar level falls towards the end of the gestation period, but rather that it is far more likely to be lowered by adverse conditions. In all cases the sheep had been brought in from pasture and transported 3 miles by lorry 2 or 3 days before that of operation. The change of environment frequently put them off their food for 1 or 2 days, which, while it would not affect the blood-sugar level of sheep out of pregnancy or those in the earlier stages of pregnancy, did cause a lowering of the level in sheep heavy in lamb. But if such sheep were not operated on and so given an opportunity to become accustomed to their new surroundings, as indicated by a resumption of normal feeding, their blood sugar subsequently rose to normal levels. It is, perhaps, no coincidence that the glycogen content of the maternal liver in sheep 758 (132 days pregnant) was nil, but whether the ratios given in Table 10 for this sheep and sheep 750 would have been the same if the maternal glucose level had been normal is unpredictable.

Table 9 also shows the differences in the glucose levels of the uterine vessels and the fructose and glucose levels of the umbilical vessels. The mean loss of glucose from the maternal blood is 2·86 mg. % with a standard deviation from

TABLE 10. Glucose levels in the umbilical vein and uterine artery

Foetal age (days) ...	64	74	85	99	100	116	132	145
Glucose (mg. %)								
(*a*) Umbilical vein	13	11	19	23	31	35	8	11
(*b*) Uterine artery	45	44	36	41	41	36	16	21
(*a*) As percentage of (*b*)	29	25	53	56	76	97	50	52

the mean of 0·91, while the mean gain by foetal blood passing through the placenta is 2·67 mg. % with a standard deviation from the mean of 1·31. The loss of glucose by the uterine artery is significant, since the possibility of obtaining a mean difference of zero is almost exactly 0·01, but the gain of glucose by the umbilical vein is not significant, the corresponding figure being rather greater than 0·05. On the other hand, the probability that the combination between the observed means of the umbilical and uterine vessels, which are of opposite sign, is due to chance, is so low (0·004) that the combination must be taken to mean that the exchange is significantly different from zero. It can be seen from the table that the differences in the fructose levels of the umbilical vessels are inconsistent.

The concentration of fructose in the maternal blood is given to the nearest mg. %. By correcting for the glucose present true levels of fructose in the two vessels can be obtained. These figures are given in Table 11 and are seen to fall towards a normal level of 0·4 mg. % with progress of the gestation period.

TABLE 11. True fructose levels in uterine artery and vein

Sheep no. ...	722	723	724	725	729	728	734	758	750	753
Foetal age (days) ...	60	64	74	85	99	100	116	132	145	146
Fructose (mg. %)										
Uterine artery	2·8	1·2	1·4	—	1·1	0·9	0·9	0·5	0·6	0·4
Uterine vein	—	2·0	1·4	0·5	—	0·9	0·5	0·7	0·7	—

DISTRIBUTION OF SUGARS IN FOETAL BLOOD

The results of four determinations on blood from the foetal carotid are shown in Table 12. The concentrations are expressed as mg. sugar per 100 ml. whole blood, per 100 ml. corpuscles, or per 100 ml. plasma as the case may be. The ratio of the concentration of fructose in the corpuscles to that in the plasma is shown to vary from 73 to 84 % in foetuses from 99 to 132 days old. For glucose the ratios are not so constant, but there is always more in the corpuscles than in the plasma, in one instance more than three times as much. The ratios for total sugar are less variable and indicate an approximately even distribution. For comparison, the distribution of total sugar was followed in three lambs and these results are given in Table 13.

Table 12. Distribution of sugars in blood from the foetal carotid

Sheep no.	729			731		
Foetal age (days)	99			106		
Haematocrit value	34			40		
	Total sugar (mg. %)	Fructose (mg. %)	Glucose (mg. %)	Total sugar (mg. %)	Fructose (mg. %)	Glucose (mg. %)
Whole blood	114	81	33	111	81	30
Corpuscles (*a*)	126	65	61	105	73	32
Plasma (*b*)	108	89	19	115	87	28
(*a*)/(*b*) × 100	117	73	320	95	84	114
Sheep no.	733			758		
Foetal age (days)	127			132		
Haematocrit value	42			47		
	Total sugar (mg. %)	Fructose (mg. %)	Glucose (mg. %)	Total sugar (mg. %)	Fructose (mg. %)	Glucose (mg. %)
Whole blood	99	72	27	68	57	11
Corpuscles (*a*)	93	60	33	66	51	15
Plasma (*b*)	103	81	22	70	63	7
(*a*)/(*b*) × 100	90	74	150	94	81	210

Table 13. Distribution of total sugar in blood from the jugular vein of lambs

Lamb	Age (days)	Haematocrit value	Corpuscles total sugar (mg. %) = (*a*)	Plasma total sugar (mg. %) = (*b*)	Whole blood total sugar (mg. %)	(*a*)/(*b*) × 100
1	15	48	81	113	98	72
	29	42	37	80	62	46
	52	34	17	59	45	29
2	15	49	66	67	67	99
	26	40	28	75	56	37
	52	32	23	90	69	26
3	8	54	48	103	73	47
	21	50	34	75	55	45
	54	39	22	82	58	26

DISCUSSION

Uptake of glucose by the foetal blood. The gain of glucose by the foetal blood can be compared with that of oxygen. The gain varied from 2 to 8 mg. % in the period of pregnancy from 85 to 132 days, while the excess of oxygen in the umbilical vein over that in the umbilical artery was found in the same period to vary from 2·5 to 7 c.c./100 c.c. blood (Barcroft, 1946*a*). In terms of oxygen required for complete oxidation, the gain of glucose can be stated as varying from 1·5 to 6 c.c. The two ranges are very close and suggest that no factors other than the oxidation of glucose are responsible for the oxygen used by the foetus. In support of this is the probability, as shown by the figures in Table 9, that no fructose is gained by the foetal blood traversing the placenta. On the other hand, a small loss of lactic acid is indicated by the figures given in Table 14, which relate to sheep from the earlier season (quoted by courtesy of S. W. Cole).

The uptake of glucose by the foetal blood remained constant in spite of the reduction in the gradient between the maternal and foetal bloods (Table 10) during the period 64–116 days through the raising of the level in the latter to that in the former. The decrease in the gradient might have been due to increase in the permeability of the placenta to glucose. But over the same period, leakage of fructose into the maternal circulation diminished (Table 11). Furthermore, the figures for oxygen pressures in the uterine artery and umbilical vein showed a widening disparity from 81 days onward (Barcroft, 1946*b*).

TABLE 14. Lactic acid in foetal blood traversing the placenta (mg. %)

Sheep no. ...	627	631	630	635
Foetal age (days) ...	102	102	110	122
Umbilical vein	18	13	26	16
Umbilical artery	18	17	27	17

Consequently, it seems more likely that the low level of glucose in the foetal blood was due to that sugar being actively withdrawn from the circulation within the foetus by a process which became less pronounced as pregnancy proceeded. The amount of glucose lost to the foetal fluids from 60 days onwards is indicated by the fact that the total volume of the amniotic and allantoic fluids amounts to about 500 c.c. at 60 days pregnant (of which the allantoic fluid accounts for one-fifth and remains approximately constant thereafter (Paton *et al.* 1907), while the concentration of glucose shows no tendency to increase. (Cole & Hitchcock, 1946).

Distribution of sugars in foetal blood. The ratio in percentage of total sugar in the foetal corpuscles to that in the plasma varied from 90 to 117 in the later stages of pregnancy, whereas Somogyi (1933) found ratios of 9 and 16 % in the normal adult and Svedberg (1933) found a variation of 8–12 % in rams. The observations made on lambs showed the distribution ratios to be falling in the first month of life towards the adult level. But useful interpretations of these comparisons and of those with respect to the distribution ratios for fructose and glucose cannot be made in the absence of a knowledge of the water contents of the corpuscle.

Origin of fructose. The determinations of the levels of fructose and total sugar in the blood of the umbilical vessels and the estimation of the distribution of glucose and fructose between the corpuscles and plasma of the foetal blood were made partly with the idea that the results might indicate the site of formation of fructose. But no consistent uptake of fructose by the umbilical vein during the second half of pregnancy could be demonstrated, and the results for the distribution of the sugars gave no information on this point.

Consequently, a more direct means of demonstrating the site of origin of fructose was attempted. It was thought possible that fructose might be formed by the breakdown of glycogen in those tissues in which it was present

in high concentration, during incubation, just as the glycogen in the adult liver is partly converted to glucose under similar conditions. Now Szendi (1934, 1936) had shown that the uterus, the placenta, the foetal lung and the foetal liver were all sites of high concentrations of glycogen in man and rabbit; all these organs showed peak concentrations at various stages in the gestation period, the peak for the uterus being followed by that for the placenta; then came the peak for the lung and finally that for the foetal liver was reached at birth. All these tissues were accordingly investigated for glycogen and incubated at 37° C.

In the case of the uterus and placenta, determination of glycogen never showed concentrations higher than 65 mg. % in either of these organs taken from ewes which had been pregnant for 47 days or more. Determinations on the foetal lung indicated a peak concentration of glycogen of approximately 1 % at 114 days, and Aron (1922) had shown that the glycogen of the foetal liver in sheep rose sharply from very low levels around 115th day of pregnancy. But no increase in fructose concentration was ever found in the liver after incubation for 1 hr. at 37° C., and there was never an outstanding rise in the foetal lung. However, fructose has to be produced throughout the second half of pregnancy, since the level in the foetal blood is not reduced to the same extent as the blood is reduced in volume; while the fructose concentration in the umbilical vein fell from 102 mg. % at 85 days to 58 mg. % at 132 days, the volume of blood rose from 60 c.c. at 85 days to 450 c.c. at 135 days (Barcroft, 1946c). But the concentration of fructose in the amniotic fluid showed no tendency to fall in the later stages of pregnancy (Cole & Hitchcock, 1946) when the volume of foetal fluids is constant, and, furthermore, the concentrations of fructose in the uterus, placenta, foetal lung and liver were never found to be higher than that in the foetal blood.

SUMMARY

1. The levels of fructose and glucose in the foetal and maternal bloods of sheep were followed during the second half of the gestation period. The level of fructose in the foetal blood was found to fall towards term and that of glucose to rise from a low level to one normal for the maternal blood.

2. A constant loss of glucose by the maternal blood traversing the placenta was demonstrated and found to be equivalent, in qualitative terms, to the average gain by the foetal blood.

3. The concentration of fructose in the maternal blood was always insignificant as compared to that in the foetal blood.

4. The distribution of total sugar between the corpuscles and plasma in the blood of foetal sheep during the latter third of pregnancy was found to be uniform; a higher concentration of glucose in the corpuscles was balanced by their having only three-quarters the concentration of fructose found in the plasma.

I am indebted to the late Sir Joseph Barcroft for his constant help and encouragement.

REFERENCES

Aron, M. (1922). *Bull. Soc. Chim. biol., Paris*, **4**, 209.
Barcroft, J. (1946*a*). *Researches on Pre-natal Life*, p. 196. Oxford: Blackwell.
Barcroft, J. (1946*b*). *Researches on Pre-natal Life*, p. 285. Oxford: Blackwell.
Barcroft, J. (1946*c*). *Researches on Pre-natal Life*, p. 74. Oxford: Blackwell.
Bacon, J. S. D. & Bell, D. J. (1946). *Biochem. J.*, **40**, xlii.
Cole, S. W. &. Hitchcock, M. W. S. (1946). *Biochem. J.* **40**, li.
Fujita, A. & Iwatake, D. (1931). *Biochem. Z.* **242**, 43.
Hitchcock, M. W. S. & Phillipson, A. T. (1946). *J. Physiol.* **105**, 42 *P*.
Karvonen, M. J. (1948). Personal communication.
Orr, A. P. (1924). *Biochem. J.* **18**, 171.
Paton, D. N., Watson, B. P. & Kerr, J. (1907). *Trans. Roy. Soc. Edinb.* **46**, 71.
Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Somogyi, M. (1933). *J. biol Chem.* **103**, 665.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
Svedberg, A. A. (1933). *Skand. Arch. Physiol.* **66**, 113.
Szendi, B. (1934). *Arch. Gynaek.* **158**, 409.
Szendi, B. (1936). *Arch. Gynaek.* **162**, 27.
Wintrobe, M. M. (1942). *Clinical Haematology*, p. 792. Philadelphia: Lea and Febiger.

J. Physiol. (1949) 108, 127–141 612.017.4:612:842.4

THE PERIPHERAL ACTION OF *CL. BOTULINUM* TOXIN

By N. AMBACHE

From the Department of Physiology, University College, London

(*Received* 3 *February* 1948)

The nature of the paralysis which occurs in botulism does not appear to have been considered in the light of modern conceptions of humoral transmission at nerve endings. It is clear from the older work of Edmunds & Long (1923), and of Dickson & Shevky (1923) that the toxin of *Cl. botulinum* affects only those nerve-muscle and nerve-gland systems that are now described as cholinergic. These authors mapped out indirectly, with toxin, the distribution of cholinergic nerve fibres in the body, before this was done systematically by direct experiment. They thought that the essential action of the toxin was upon the motor-end plates of skeletal muscles, and, with other authors, have suggested a type of action similar to that of curare on striated, and of atropine on plain, muscle. Their experiments, however, only showed that the muscles in the paralysed limb were still excitable electrically, and did not prove the pharmocological action which they postulated; but they served to locate the paralysis at some point proximal to the muscle fibres. This question has therefore been re-examined by studying the local effects of the toxin (*a*) on the nerve-smooth muscle systems in the iris, after intraocular injections, and (*b*) on various voluntary muscles paralysed by local injections of toxin.

The results show that the toxin has no appreciable effect either on the sensory or on the adrenergic nerve fibres within the eye, but that it affects the cholinergic fibres specifically, and that this cholinergic paralysis is not due to the existence of a block between the transmitter and the effector cell.

METHODS

A powdered preparation of *Cl. botulinum* toxin type A, which was kindly supplied by Dr D. W. Henderson of the Microbiological Research Station, Porton, was used. Its mouse a.l.d. was 0·1 μg./kg. A small amount of it was dissolved in a sterile buffer of the following composition: 0·397% Na_2HPO_4, 0·3% powdered gelatin; pH adjusted to 6·6 (electrometrically) by adding 0·8–1 ml. N-HCl. To this solution an equal volume of pure glycerol was added. The glycerinated toxin, which had a final concentration of 10^{-3}, was stored in darkness in a refrigerator. Further dilutions of toxin in phosphate buffer were made up from this stock solution immediately before the injections. The pH of these dilutions was also 6·6 for the first few experiments, but it was later raised to 7·2, in order to avoid undesirable inflammatory reactions in the vitreous body.

Rabbits of mixed stock weighing 0·85–2·7 kg. were used in all the experiments on the eye. Because of the expense, an accurate determination of the m.l.d. for rabbits was not carried out but an approximate value for the m.l.d. (4 days) by the intravenous route (*c.* 0·05 μg./kg.) was arrived at from the five experiments quoted in Table 3 although the exact death-time was not measured, as the animals were used for experiment when they were moribund. For inoculation into the eye the rabbits were anaesthetized with nembutal (usual dose: 26 mg./kg. intravenously) and the toxin was injected: (*a*) into the anterior chamber of the eye, through the cornea. The technique of injection has been previously described in detail (Ambache, Morgan & Payling Wright, (1948); the only differences have been the omission of the local anaesthetic and the withdrawal of an equal volume of aqueous humour before the injections; (*b*) into the vitreous body: for this, the eyeball was rotated downwards and a puncture was made about 4–5 mm. behind the sclero-corneal junction, the needle being driven into the eye until it was visible in the vitreous cavity at a depth of 1–1·5 cm.; (*c*) subconjunctivally and into, or, more probably, around the m. rectus superior. The volume of all these injections was usually 0·02–0·05 ml. In a few experiments the toxin was instilled drop by drop into the conjunctival sac. In each animal only one eye received active toxin. The other eye, which served as a control, was either left without interference or received an identical volume of the same toxin solutio , boiled for at least 2 min.

The horizontal diameters of the pupils were measured with a pair of calipers. Reaction to light was tested with a 60 W. lamp at 4–5 cm. distance from the eye, with a few exceptions in which bright sunshine was available on a few cloudless days in September. This was a more powerful stimulus and was used whenever possible. For faradic stimulation of the oculomotor nerves these were exposed in their cranial portion, after decerebration (under nembutal) and section of the optic nerves (Ambache, Morgan & Payling Wright, 1948). Stimuli of measured duration were delivered from a Du Bois-Reymond induction coil (turns ratio: 25/1) with a 2 V. battery in the primary.

For recording the contractions of the m. rectus superior, the conjunctiva was incised circularly round the sclerocorneal margin and reflected backwards. A thread was tied round the tendon of the superior rectus, which was severed: (*a*) from its insertion into the eyeball, (*b*) from the tendon of the superior oblique which adjoins it. The eyeball was freed, by dissection, from the other muscles which are inserted into it. Then, in order to minimize lateral friction on the superior rectus, the eyeball was either wholly eviscerated after preliminary ligation of the ophthalmic artery and optic nerve, or it was simply collapsed by making a circumferential cut and removing the anterior half of the eye together with the vitreous body in the posterior half. For myographic recording, the animal's head was held rigidly in a suitable head-clamp, and was further immobilized either by screwing the threaded end of a brass rod into a hole drilled in the occipital bone and fixing the rod to a metal bar on the operating table, or by means of a second clamp. The two superior recti muscles were connected in turn to the same sensitive torsion myograph for recording tensions. The common carotid arteries were needled for the arterial injections of acetylcholine.

Experiments on tibialis anticus. For these experiments cats were used. They were anaesthetized with ether and the skin was shaved over the tibialis anticus muscle on one or both sides. 5–50 μg. of toxin, in a total volume of 1 ml. saline, were injected on one side, in divided amounts, into the tibialis anticus muscle at 16–20 points down its whole length. The control muscle was injected in three out of seven of these experiments in exactly the same way with an identical amount of toxin boiled previously for 2 min.

Two to eight days later the animals were either decerebrated under ether or anaesthetized with nembutal (26–38 mg./kg. intraperitoneally) and the two tibialis muscles were prepared for myographic recording. The tendons of the extensor digitorum longus and of the peroneal muscles were cut. The peroneal nerve was dissected and prepared for stimulation where it curves round the head of the fibula, after placing a tight ligature round the whole sciatic nerve high up in the thigh. For stimulation neon discharges were led in through a transformer to shielded electrodes which were placed under the nerve. The frequency of stimulation varied between 1 in 10 sec. and 1 in 12·5 sec.

The paralysed muscle was then prepared for close arterial injection, the procedure described by Brown (1938) being followed closely. Acetylcholine was injected in doses of 2–20 μg., in a volume of 0·2–0·5 ml. saline.

RESULTS

Intraocular changes induced by the toxin

Loss of reaction to light. As shown in Table 1, which is summarized from more than forty measurements on 18 animals, the well-known botulinic effect on the iris is only obtained (within 24–48 hr. usually) with doses of toxin which approximate to or exceed the intravenous m.l.d. In order to abolish completely the reaction to light it was necessary to inject 0·05–0·1 μg. of toxin into the vitreous body and still larger doses into the anterior chamber. With amounts smaller than this a total paralysis was rare, although there were varying degrees of impairment of the light reflex, which was also more sluggish than in the control eye. Boiling the toxin for 2 min. for the control injections, which was done in five experiments, completely destroyed its activity in every case.

TABLE 1. Changes in the reaction to light in rabbits after intraocular injections of *Cl. botulinum toxin*

Dose of toxin (μg.)	Time of measurement after inoculation	Reduction in the horizontal diameter (mm.) of the pupil on illumination† (the number of observations is given in brackets wherever an average has been taken): Control eye‡	Intoxicated eye
	A. Into the vitreous body		
0·01–0·05 (3 animals)	—	—	Weak reactions
0·1	48 hr.	2·5*	0*
0·1	72 hr.	1·5	0
0·2	68 hr.	2·25*	0*
0·5	19 hr.	2·5	3.5
	47 and 70 hr.	2·25 (2)	0 (2)
1	42 hr.	3*	0*
5	22 hr.	2	0·25
	B. Into the anterior chamber		
0·3–0·5 (4 animals)	—	—	Weak reactions
1	19 hr., 2, 3, 4 and 11 days	2·15 (5)	0 (5)
	16 days	2·25	1
1·2	72 hr.	1·5	1
1·5	45 hr.	4*	1·5*
2	24 hr.	1·5	0
2·5	20, 44 hr. and 3 days	2·5 (3)	0·4 (4)
	9 days	2·5	1·75

† For illumination a 60 W. lamp was used at a distance of 1–5 cm. from the eye, except for the measurements marked with an *asterisk*, which were made in direct sunlight.
‡ In five experiments the control eye received boiled toxin; in the others it was untreated.

Size of the pupil. Changes in pupillary diameter may or may not be concurrent with the loss of reaction to light in rabbits. All the measurements taken within 24 hr. of inoculation showed a difference in size between the two pupils,

with mydriasis present on the intoxicated side. Subsequently the pupils tended to become equal, although the mydriasis perisisted in a few experiments and a slight myosis on the intoxicated side was recorded twice.

Response to nerve stimulation; effect of the toxin on different types of nerve fibre

Cervical sympathetic. In all of five experiments stimulation of the cervical sympathetic nerve dilated the pupil on the intoxicated side approximately to the same extent as on the control side, even when the reaction to light was lost completely (Table 2).

TABLE 2. Effect of the toxin, injected into the vitreous body, on the pupillary response to stimulation of the oculomotor and cervical sympathetic nerves in rabbits. − indicates constriction; + dilation. Faradic stimulation of equal intensity and 5 sec. duration was applied to each side

Dose of toxin (μg.)	Time after inoculation	Reaction to light on intoxicated side	Nerve stimulated	Pupillary response in mm. (the number of observations is given in brackets wherever an average has been taken)	
				Control side	Intoxicated side
0·05	7 days	Sluggish	Third	—	−1, delayed (2)
0·1	72 hr.	Absent	Cervical sympathetic	+1·9 (2)	+1·9 (3)
			Third	−3	−0·75 (delayed 1 min.)
				−3·25	−0·5
				−3·75	
				−3	0
				−2·5	0
				−3	
0·2	68 hr.	Absent	Cervical sympathetic	+2·75 (2)	+1·6 (4)
0·5	70 hr.	Absent	Third	−3	−0·25
				−2·75	0
				−0·5	
				0	0
				−1	0
				−0·5	
			Cervical sympathetic	+2	+2 (2)
0·5*	20 hr.	Absent	Cervical sympathetic	+2·1 (4)	+1·6 (5)
1	42 hr.	Absent	Cervical sympathetic	+2·8 (3)	+2·6 (2)

* Injection into anterior chamber instead of vitreous body. In Exps. 2 and 3 the control eye received boiled toxin; in the others it was untreated.

Paralysis of the oculomotor nerve. On the other hand, stimulation of the oculomotor nerves showed a clear-cut difference in the magnitude, and sometimes in the promptness, of the pupillary response on the two sides. For example, in the second animal listed in Table 2, the usual constriction of the pupil was obtained six times on the control side (injected with boiled toxin) and averaged 3 mm. The contractions of the sphincter started at once and were maximal at the end of the 5 sec. period of stimulation. In contrast with this, there was no response in the intoxicated eye (0·1 μg. active toxin) at the end of

stimulation, but 1 min. later it was observed that the pupil had shrunk by 0·75 mm. The next stimulus was even less effective and two others subsequently had no effect at all; the average of the four measurements was −0·3 mm.

Delayed responses were observed in another experiment, in an eye inoculated with 0·05 μg. of toxin, and were timed more accurately. The first oculomotor stimulus constricted the pupil by 0·75 mm. after a delay of 25 sec.; the second, administered 6 min. later, was more effective (1·25 mm.) and with a shorter delay (10 sec.). The sluggish reaction to light, together with the delayed and diminished response to direct stimulation of the oculomotor nerve, illustrate the type of subtotal paralysis produced by insufficient doses of toxin. When the dose was raised to 0·5 μg., as in Exp. 4, listed in Table 2, the response to oculomotor stimulation on the intoxicated side was practically zero. On the control side, which was uninjected, the response to the first two stimuli averaged −2·8 mm.; subsequently there was some evidence of fatigue on this side (final average −1·3 mm.) which may possibly indicate a slight extension of the intoxication to the opposite side.

An analogous observation indicating a crossed effect of the toxin was made by van Ermengem (1897). In three rabbits, doses of toxin which eventually killed the animals in 18–24 hr. were injected into the anterior chamber on one side but produced paresis of the iris (to light) which was equal on the two sides.

The sensory fibres in the cornea. The integrity of corneal sensation was tested by applying mechanical or weak faradic stimuli to the cornea. In normal rabbits such stimuli elicit reflex retraction of the eyeball, with passive protraction of the nictitating membrane. This test was not available when the toxin was injected into the vitreous body because of the accompanying paralysis of the extrinsic muscles of the eye described below; but it was observed that the animals attempted to withdraw the whole head when the cornea was touched. However, when the toxin was injected into the anterior chamber of the eye, through the cornea, it was possible to apply this test and it was found, in all of three rabbits examined, that corneal sensation was unimpaired at a time when the paralysis of the third nerve was fully developed.

Unimpaired contractility of the sphincter pupillae. An indication of the functional state of the sphincter muscle in the intoxicated eyes could be obtained from a study of its response to acetylcholine injected into the anterior chamber. This experiment was performed on the second to the fourth day of the intoxication, on five rabbits, two of which were not reacting to light at all and the other three hardly at all (responses of 0·5–1 mm. at most). In all of six experiments on these five animals, the injection of 0·2–2·5 μg. of acetylcholine-HCl into the anterior chamber produced a vigorous myotic contraction of the sphincter pupillae. After the same amount of acetylcholine was injected into the control eye, the final size of the pupil was equal on the two sides. One of these experiments may be quoted in detail to illustrate this point.

6 October 1947. Rabbit, 1·03 kg. Paracentesis of the anterior chamber of the left eye performed under nembutal anaesthesia (30 mg. intravenously). Puncture through the cornea 3 mm. anterior to the sclerocorneal junction. About 0·15 ml. aqueous humour withdrawn into a syringe. Without withdrawing the syringe needle from the eye, the syringe was changed for another containing the toxin. 1 mg. of toxin in 0·04 ml. sterile phosphate diluent injected. Right control eye uninjected.

9 October 1947. No reaction to light in the left eye (see Table 1). 2.36 p.m., 30 mg. of nembutal intravenously. 2.49 p.m., left pupil: 5·5 mm. 2 μg. of acetylcholine in 0·2 c.c. of sterile saline injected into the left anterior chamber. Subsequent measurements: after 60 sec., 2·5 mm.; after 2 and 4 min., 2 mm. 2.54 p.m., right pupil (control), 6·5 mm. 2·55 p.m., 2 μg. of acetylcholine injected into right anterior chamber. Subsequent measurements: after 45 and 75 sec., 2 mm.; after 2 min., 2 mm.

After each acetylcholine injection in this and some of the other experiments, the syringe needle was not withdrawn from the eye for 45–60 sec., and the first measurements of the acetylcholine effects were taken with the needle still in the anterior chamber. The reason for this is as follows. It is known that the fall in intraocular pressure which is produced by paracentesis is followed by myosis due to congestion of the iris. It was therefore essential to be certain that the response to acetylcholine occurred *before* any fluid leaked out of the eye; by leaving the needle in for a minute it was possible to show that the response of the sphincter pupillae did occur before the leak. The measurements showed that the acetylcholine effect was, 30–60 sec. after injection, only 0·5 mm. short of its eventual maximum.

These experiments show that there is no decrease in sensitivity to acetylcholine in botulism of the eye. The muscle fibres of the sphincter pupillae appear to be unaffected and the toxin does not block the action of acetylcholine in the manner of atropine.

The vitreous body and the retina. After injections into the vitreous body there was an opacity within the eye. Ophthalmoscopic examination showed that the opacity was inside the vitreous body. There was also a small unabsorbed haematoma and a patch of choroido-retinal atrophy opposite the site of injection, in the eyes injected with toxin whether active or boiled. These reactions may have been caused by the trauma of injection.

Paralysis of the extrinsic muscles of the eye

Ophthalmoplegic symptoms were first observed after inoculations of the vitreous, but were later reproduced by injecting active toxin subconjunctivally. In the first case, the paralysis appears to be caused by a leakage of toxin out of the eye. When the syringe needle was withdrawn from the vitreous cavity of the eye a leakage of fluid out of the eyeball was observed in every instance. This fluid was trapped subconjunctivally, where, within a few minutes, it formed a small bleb, followed sometimes by the appearance of conjunctival oedema in the upper fornix. In two experiments, in which indian ink was mixed with the toxin and the needle was inserted into the vitreous body to a depth of 1–1·5 cm., the indian ink did not appear in the subconjunctival bleb at any time. Therefore it seems that the bulk of the injection does not leak out of the eye at once, for the indian ink at least is trapped in the vitreous. However, judging by the paralysis of the extrinsic muscles of the eye, the molecules of toxin are able to diffuse out along this path into the tissues of the orbit.

The changes which indicated a physiological action of the toxin inside the orbit were: (*a*) paresis of the m. levator palpebrae superioris with a resulting droop of the upper eyelid and a narrowing of the palpebral fissure; (*b*) paralysis of the m. retractor bulbi. This is a striated muscle which is supplied by the oculomotor nerve (Krause, 1884); as it retracts the eyeball, the nictitating membrane is drawn forward passively across the eye. In the intoxicated orbits the eye was slightly proptosed, and active retraction of the eyeball and reflex nictitation in response to corneal stimulation were abolished. In severe cases the absence of blinking led to the appearance over the cornea of white threads of stringy mucus. The cornea itself had a dry appearance, which may indicate some impairment of the secretion of tears.

Third-nerve stimulation. When the third nerve is stimulated in normal rabbits there is, besides the usual lateral and rotatory movement of the eye, visible retraction of the eyeball and nictitation. In these experiments the skin over the cranium and upper eyelid was removed prior to decerebration and, when viewed from above, it was seen that each time the eyeball was retracted a pad of orbital fat was forced out, as a bulge upwards, into the space between the frontal bone medially and its supraorbital process laterally.

These responses to third nerve stimulation were always present on the control sides, whether uninjected or injected with boiled toxin, but with adequate doses of active toxin they were all abolished. In a few experiments it was possible to produce this type of 'local' botulism without the appearance of generalized symptoms, by subconjunctival injections of 0·02–0·12 μg. of toxin. When larger doses of toxin were used it was necessary to perform the experiments within 24–48 hr., when the symptoms were still localized to the site of injection.

Analysis of the botulinic effect. In preliminary experiments it was noticed that the 'paralysed' extrinsic muscles of the eye would still retract the eyeball when acetylcholine (0·3–0·4 mg.) was injected into the homolateral common carotid artery. Further investigations were restricted to one of the extrinsic ocular muscles, namely the m. rectus superior. For these experiments 0·2–1·5 μg. of toxin was injected subconjunctivally either into this muscle or, more probably, around it in five rabbits. On the next day there were symptoms of paralysis in the upper group of extrinsic ocular muscles. Corneal stimulation elicited only slight downward rotation of the eyeball. In one of these animals it was noticed that the cornea had a dry appearance.

When tension records were taken from the m. rectus superior, it was found, on stimulating the oculomotor nerve, that the paralysis was incomplete in two of these animals. It was evident, in one of these, that the dose of toxin had been too small (0·2 μg. injected 20 hr. previously) and, in the other, that the toxin had not been allowed to act for a sufficiently long time (24 hr. after 0·6 μg.).

In the other three experiments, which were performed 27–43 hr. after the injection of 0·3–1·5 μg. of toxin on one side, a comparison of the myograms from the two superior recti showed that the paralysis was complete on the intoxicated side. Thus, faradic stimulation of the oculomotor nerve on the control side elicited repeatable tetanic contractions of the m. rectus superior on that side (Fig. 1 A and 2 A). In contrast, faradic stimulation of identical intensity applied to the nerve on the intoxicated side was repeatedly ineffective (Fig. 1 B and C; Fig. 2 C). A small contraction was obtained, in Fig. 1 D, when the electrodes were applied directly to the muscle.

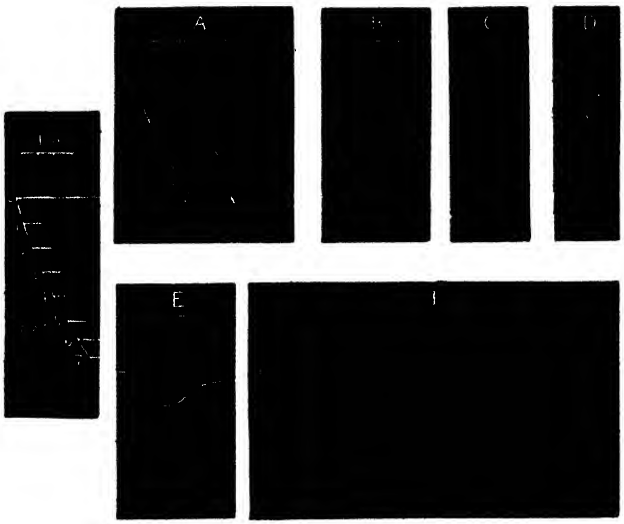


Fig. 1. Rabbit, 1·8 kg. 28 hr. after a local injection of 0·3 μg. of botulinum toxin round the left m. rectus superior. Decerebrated under nembutal (63 mg.); 2·5 mg. of atropine. Isometric myograms of the two recti superiores: A and E from the normal side; B, C, D and F from the intoxicated side. A, Three contractions of the normal muscle on electrical stimulation of the right oculomotor nerve (secondary coil at 7 cm.). B and C, An identical stimulus is applied twice to the left oculomotor nerve. The botulinic effect is fully developed and there is no response from the intoxicated muscle, which is however, contracted by direct electrical stimulation (at D). E and F, Effect of homolateral intracarotid injections of acetylcholine (300 μg.; pH_4). E, Normal muscle. F, Intoxicated muscle. Calibration in g.; the line of zero tension is shown at the top of each tracing. Time in 1 sec.

In all three of these experiments the paralysed muscles responded to acetylcholine. The animals were under the influence of atropine throughout the experiments, in order to prevent fatalities due to the 'muscarinic' action of acetylcholine on the heart, but as shown by Duke-Elder & Duke-Elder (1930) atropine does not interfere with the 'nicotinic' action of acetylcholine on the extrinsic ocular muscles.

In the first experiment a small contraction (1·3 g.) was recorded from the paralysed rectus superior muscle after the injection of 0·55 mg. of acetylcholine into the marginal ear vein. In the next experiment 1 mg. of acetylcholine (pH 4) intravenously was ineffective, but 0·3 mg. injected into the homolateral common carotid artery produced a contraction (5 g.) of the 'paralysed' muscle (Fig. 1 F) which was larger than the response to the same dose of acetylcholine

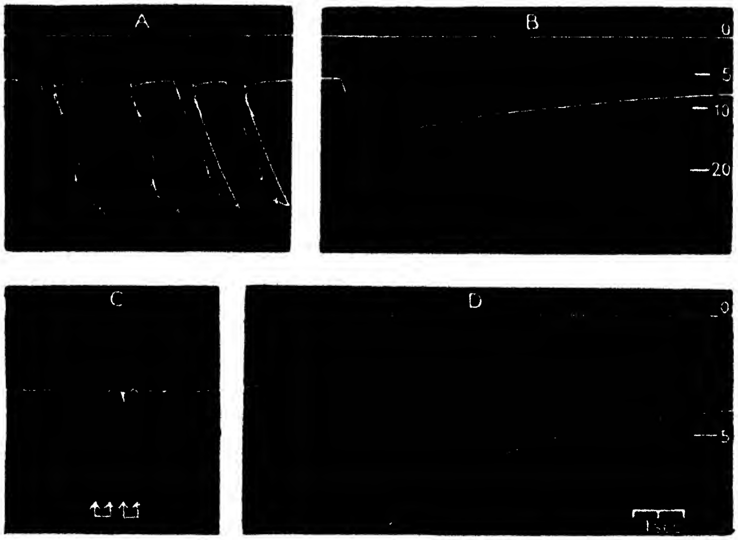


Fig. 2. Rabbit, 2·7 kg. 48 hr. after a local injection of 1·5 μg. of botulinum toxin round the m. rectus superior on one side only. Decerebrated under nembutal (78 mg.); 2 mg. atropine. *Control side.* A, Four responses to oculomotor nerve stimulation (secondary coil at 7 cm.). B, Effect of 1 mg. neutral acetylcholine injected into homolateral common carotid artery. Calibration in g. for A and B. *Intoxicated side*: (sensitivity of the lever increased in order to detect smaller responses). C, Ineffectiveness of oculomotor nerve stimulation repeated twice (secondary coil at 7 cm.). D, Response to 1 mg. neutral acetylcholine injected into homolateral common carotid artery. Calibration in g. for C and D. Line of zero tension shown at the top of each tracing. Time in 1 sec.

on the sound side (Fig. 1 E). In the last experiment (Fig. 2 D) 1 mg. of neutral acetylcholine, also intra-arterially, again produced contractions of the paralysed muscle, but this time the contractions were slightly smaller (6–7 g.) than the response (8 g.) to the same dose of acetylcholine on the opposite side.

Confirmatory experiments on the tibialis anticus muscle (cats)

Since this paper was submitted for publication an earlier paper by Guyton & MacDonald (1947) has come to the author's notice. The results described in this section are in agreement with their observations on the gastrocnemius in rabbits and guinea-pigs, and with those of Burgen, Dickens & Zatman (1948) on the rat's phrenic-diaphragm preparation.

Cats are able to tolerate larger doses of botulinum toxin, and one of the animals in this series survived for 8 days after it had received 50 μg. into one of its tibialis anticus muscles. The majority of these experiments, however, were performed on the third day after the intra-muscular injection. In every case it was found that, in doses of 10, 40, and 50 μg. the toxin had produced a total paralysis of the tibialis anticus (see Fig. 3 B), whereas the muscle on the control side (injected with the same amount of boiled toxin in three of these experiments) responded normally to peroneal nerve stimulation (Fig. 3 A). In

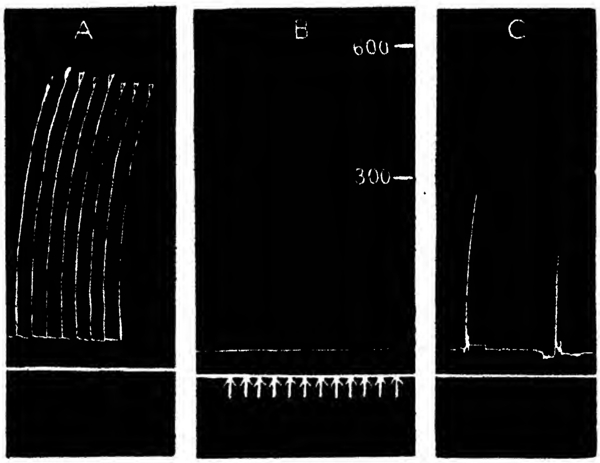


Fig. 3. Cat, 2·7 kg. Injected 3 days previously with 50 μg. of botulinum toxin into the right, and with the same amount of boiled toxin into the left, tibialis anticus. Anaesthetized with 105 mg. of nembutal intraperitoneally (and subsequent maintenance doses totalling 49·5 mg. over the 4 hr. of the experiments). A, Left tibialis (*control*). Twitches elicited by supramaximal stimulation of the left peroneal nerve once every 12·5 sec. B and C, From right tibialis (*intoxicated side*). B, Twelve stimuli of identical intensity and frequency were applied to the right peroneal nerve at the arrows; showing the muscle to be totally paralysed. C, Effect of close arterial injection of 10 μg. of acetylcholine, repeated twice. Bottom line indicates zero tension in all the records. Calibration in g. shown in B.

another experiment 10 μg. produced complete paralysis within 46 hr.; it is probable that this dose of toxin is effective within a much shorter time interval, but no experiments were performed to determine this. With 5 μg. of toxin paralysis was total after 45 hr. in one experiment; in a second cat, 45 hr. after the same dose, there were still measureable responses from the intoxicated muscle on motor-nerve stimulation, but the tension developed was only 25–45 g. as compared with 750 g. on the opposite side. It was noticed, in some of these experiments, that although there was no response from the intoxicated tibialis anticus on stimulating the peroneal nerve, there was, however, synchronously with each neon discharge during the period of stimulation, an extension of the toes on the same side. This appears to be due to excitation of the

m. extensor digitorum brevis, which is supplied by the same nerve lower down.

In four of these experiments, in which total paralysis had been obtained, a twitch could be elicited from the 'paralysed' muscle by close arterial injections of acetylcholine. One of these experiments is illustrated in Fig. 3, where the effect of two successive injections of 10 μg. of acetylcholine is shown at C.

These and the experiments in the preceding section show quite clearly that in somatic botulism, the voluntary muscle fibres, although effectively paralysed, can still react to acetylcholine. There is thus no similarity between this type of paralysis and that produced by curare.

Systemic effects (rabbits)

Intravenous injections. An approximate idea of the m.l.d. by this route can be gained from Table 3. All but one of the animals developed the classical symptoms of botulism. In addition to paresis and flaccidity of the muscles in the limbs, the neck and the abdominal wall, there was considerable salivation and the appearance of moisture round the nostrils. When the animals were moribund, the heart rate tended to be rather irregular and slow. Sluggishness of the pupil was seen in only one animal. At autopsy the lungs presented a consolidated, haemorrhagic appearance. Histological sections of these lungs showed that the consolidation was due to oedema and congestion; there were also areas of scattered haemorrhages into the substance of the lungs. The alveolar exudate contained no fibrin, polymorphs or red cells, which distinguishes these botulinic lesions from the pathological changes found in pneumonic consolidation.

Vitreous body injections. In view of the leakage of toxin out of the eye which has been mentioned, it is not surprising that some of the animals injected by this route developed symptoms of general botulism. But in order to produce the same effect approximately ten times as much toxin is required as by the intravenous route. The lung changes described above were found in one rabbit, (3·4 μg. toxin).

Conjunctival instillation. It is known that rabbits are relatively resistant to botulinum toxin by ingestion, but for reasons which will be apparent in the next paragraph, it was necessary to know whether the toxin is absorbed from the conjunctival sac, and the nasolachrymal duct. Very large amounts of toxin (4·54, 8 and 44·4 μg/kg. respectively) were therefore instilled into one or both conjunctival sacs in three rabbits. In all three there were no observable symptoms of botulism, either local or general, and the animals were still alive and well 16 days later. This confirms an old observation of van Ermengem (1897) in which, however, the amounts of toxin used were not specified.

Injections into the anterior chamber. After the injections a certain amount of aqueous humour was seen leaking out into the conjunctival sac, but as we have

just seen, the toxin is not absorbed through the conjunctiva. Therefore, if any systemic effect is produced by this route, it must indicate an absorption of toxin from the anterior chamber of the eye. With appropriate doses of toxin (see Table 3) this was found to be the case and the animals died with general

TABLE 3. Occurrence of systemic effects in rabbits (see text)

Route of injection	Dose of toxin (µg./kg.)	Time when general symptoms were first observed	Subsequent course	Findings at autopsy
Intravenous	0·035	—	Alive and well 16 days later	—
	0·045	70 hr.	Died between 80 and 92 hr.	Mild haemorrhagic lung lesions (confirmed histologically)
	0·05	45 hr.	Killed at 4 days (moribund)	Autopsy not performed
	0·08	45 hr.	Killed at 49 hr. (moribund)	Characteristic lung lesions
	0·25	19 hr.	Killed at 24 hr (moribund)	Lung lesions: oedema (confirmed histologically)
Anterior chamber	0·25	4 days	Killed at 6 days (moribund)	Lung lesions
	0·26	—	—	—
	0·29	—	Killed at 19 hr.	Autopsy not performed
	0·29	3rd day	Died between 3rd and 5th day	,, ,, ,,
	1	—	Alive and well 11 days later without general symptoms	—
	1·5	—	Alive and well 14 days later	—
	1·66	50 hr.	Died between 50 and 65½ hr.	Characteristic lung lesions (confirmed histologically)
	2·35*	—	Died between 24 and 65 hr.	,, ,,
	2·77	—	Alive and without general symptoms 9 days later	—
	3·37	—	Died in 18 hr.	No autopsy
Vitreous body	0·007 to 0·11 (6 rabbits)	—	—	—
	0·15	68 hr.	Killed at 68 hr.	Lungs appear normal at autopsy
	0·31	—	Killed at 70 hr.	,, ,,
	0·88	—	Killed at 42 hr.	No autopsy
	2·43	—	Died at 20 hr.	,,
	3·4	24 hr.	Died between 29 and 44 hr.	Characteristic lung lesions
Conjunctival instillation	4·54	—	Alive and well 16 days later. No symptoms then or subsequently	
	8	—	,, ,,	—
	44·4	—	,, ,,	—

* Half this dose was injected into the anterior chamber proper. The other half accidentally into the substance of the cornea itself.

symptoms of paralysis and with characteristic lesions in the lungs. The presence of oedema was confirmed histologically in two of these animals. It would seem therefore that despite its very large molecular weight (1,130,000) the toxin can permeate out of the eye into the general circulation.

DISCUSSION

The results show that *Cl. botulinum* provides us with a neurotoxin which acts specifically on the nerve fibres of the cholinergic group. In one and the same organ, the adrenergic and sensory nerve fibres are relatively unaffected by it, although they have been exposed to exactly the same concentrations of toxin, within the eye, which are adequate to paralyse the cholinergic fibres. There is, therefore, no reason to believe that the toxin interrupts the conduction of nervous impulses, unless we are to believe that the basis of nervous conduction in cholinergic fibres is intrinsically different from that of other nerve fibres in the body, for which there is no evidence. And indeed, in several preliminary experiments in which 5–20 μg. of toxin have been injected within the sheath of the sciatic nerve well above its bifurcation, it has been found, that stimulation of that nerve 2–3 days later still elicits as usual vigorous contractions of the tibialis anticus (in cats). It is therefore, more probable that the specific action on the fibres of the cholinergic group is located at the nerve endings and that the toxin interferes with the process of humoral transmission. The suggestion that this interference is of the nature of a 'block', such as is seen after curarization or with atropine, receives no support from these experiments; on the contrary, it is evident that the muscle fibres, both plain and striped, respond vigorously to acetylcholine. It is more probable, therefore, that humoral secretion is at fault.

This neurotoxic effect shows a certain resemblance to the paralytic action of tetanus toxin previously described (Ambache, *et al.*, 1948). Both organisms, *Cl. tetani* and *Cl. botulinum*, are fairly close members of an anaerobic group of bacteria. It is perhaps worth stressing here that 'tetanic' symptoms may occur in botulism (convulsions and salivation) and botulinic symptoms in tetanus (ophthalmoplegia, and paralysis of the lingual and facial nerves). Despite the great difference in the molecular weight of these two bacterial products, it may be that their active parts, or the toxic derivatives produced from them within the body, are more closely related than was suspected.

One may perhaps make one further inference from these experiments. The molecular weight of botulinum toxin, which has been isolated in crystalline form as a single protein, has been found to be of the order of 900,000 to 1,130,000 (Kegeles, 1946; Putnam, Lamanna & Sharp, 1946). It is difficult to see how a molecule of this size could (*a*) be absorbed from the gut, (*b*) pass out of the blood stream through an intact capillary endothelium to reach the cholinergic nerve endings, and (*c*) escape out of the eye after anterior chamber injections. It appeared possible that the toxin was capable of producing a necessary change in membrane permeability by possessing a lecithinase activity as shown by Owen, Langohr & Blakely (1947) who used a crude toxin preparation. This might, for example, have accounted for the severe oedema of the lungs which

was found at autopsy. However, the toxin preparation which was used for these experiments was tested (Rees, 1948) by the lecithovitellin reaction and did not display any lecithinase activity. Nevertheless, it is possible that the toxin can, in some other way, induce a change in capillary permeability permitting its passage through the capillary membrane. Alternatively, or in addition, the original bacterial product may be a 'pro-toxin' which, when it enters the living organism, is broken down to smaller units of diffusible size which are still neurotoxic. In this connexion it was shown by Schübel (1923) that the toxin, although a protein, can withstand both peptic and tryptic digestion without loss of activity. It is also known (Krause, 1934) that both the anterior chamber and the vitreous body contain a protease, which might break down such a protoxin before it can escape out of the eye.

SUMMARY

1. The local effects produced by the intraocular injection of *Cl. botulinum* type A toxin have been studied in rabbits.

2. In appropriate doses the toxin produces a total paralysis of the cholinergic nerve fibres to the sphincter pupillae, which fails to react to light or to oculomotor nerve stimulation. The muscle fibres in the sphincter still respond at this stage to acetylcholine in small doses.

3. The adrenergic fibres within the eye are relatively unaffected by the toxin, and stimulation of the cervical sympathetic nerve dilates the pupil as usual.

4. The function of the sensory nerve fibres in the cornea appears to be intact and, if the toxin is injected into the anterior chamber of the eye, reflex blinking and retraction of the eyeball can still be elicited by mechanical or electrical stimulation of the cornea.

5. There is evidence that the toxin can escape into the general circulation from the anterior chamber and produce lesions in the lungs. The implications of this are discussed.

6. When injected subconjunctivally, or when it leaks out from the vitreous body, the toxin produces additional local effects on the extrinsic muscles of the eye. These include the paralysis of the m. retractor bulbi, which results in proptosis and a loss of nictitation.

7. The m. rectus superior was chosen for an analysis of the toxic effect on voluntary muscles. The 'paralysed' muscle does not respond to oculomotor nerve stimulation but is still contracted by intracarotid injections of acetylcholine.

8. These results were confirmed on the tibialis anticus m. of the cat. After local injections of toxin the muscle was completely paralysed when its motor nerve was stimulated, but responded to close arterial injections of acetylcholine.

9. These and other findings suggest that botulinum toxin exerts its paralytic action by means of a selective peripheral effect on the cholinergic nerve-endings.

I wish to express my gratitude to Dr G. L. Brown, for instructing me in the preparation of tibialis anticus, and to Prof. C. Lovatt Evans and Sir Stewart Duke-Elder for their advice and interest. My thanks are also due to Miss Jean Barrett for her help throughout this investigation, the expense of which was borne by a grant from the Medical Research Council.

REFERENCES

Ambache, N., Morgan, R. S. & Payling Wright, G. (1948). *J. Physiol.* **107**, 45.
Brown, G. L. (1938). *J. Physiol.* **92**, 22*P*.
Burgen, A. S. V., Dickens, F. & Zatman, L. J. (1948). *J. Physiol.* **107**, 41 *P*.
Dickson, E. C. & Shevky, R. (1923). *J. exp. Med.* **37**, 711.
Duke-Elder, W. S. & Duke-Elder, P. M. (1930). *Proc. Roy. Soc. B.* **107**, 332.
Edmunds, C. W. & Long, P. H. (1923). *J. Amer. med. Ass.* **81**, 542.
van Ermengem, E. (1897). *Arch. int. Pharmacodyn.* **3**, 213, 499.
Guyton, A. C. & MacDonald, M. A. (1947). *Arch. Neurol. Psychiat., Chicago*, **57**, 578.
Kegeles, G. J. (1946). *J. Amer. chem. Soc.* **68**, 1670.
Krause, A. C. (1934). *The Biochemistry of the eye.* Baltimore: Johns Hopkins Press.
Krause, W. (1884). *Die Anatomie des Kaninchens.* Leipzig: Engelmann.
Owen, C. R., Langohr, J. L. & Blakely, E. (1947). *J. Path. Bact.* **59**, 261.
Putman, F. W., Lamanna, C & Sharp, D. G. (1946). *J. biol. Chem.* **165**, 735.
Rees, R. (1948). Unpublished observations.
Schübel, K. (1923). *Arch. exp. Path. Pharmak.* **96**, 193.

J. Physiol. (1949) 108, 142–156 612.116

THE DETERMINATION OF THE BLOOD VOLUME IN MAN BY THE CARBON MONOXIDE AND DYE METHODS

By F. C. COURTICE and R. W. GUNTON

From the University Laboratory of Physiology, Oxford

(*Received* 14 *February* 1948)

The blood volume in man has largely been determined by one of two methods, the dilution of a known volume of carbon monoxide in the corpuscles or of a known amount of dye in the plasma. Carbon monoxide was first used for this purpose in the human subject by Haldane & Smith (1900). The general procedure for the administration of the carbon monoxide is still in principle the same as that used by these investigators, but the analytical methods for determining blood carbon monoxide have improved in accuracy. Haldane employed the carmine titration method which has certain disadvantages, and is not suitable for very low concentrations in the blood. Van Slyke & Salvesen (1919) introduced a method involving the use of the van Slyke apparatus, and further improvements in this technique have been made (cp. Peters & van Slyke, 1932). More recently, Scholander & Roughton (1943) have evolved an accurate and convenient method for the determination of small amounts of carbon monoxide in blood. This requires only small quantities of blood and simple apparatus, and so enables the blood volume to be determined more easily by the carbon monoxide method.

The dye method was first introduced by Keith, Rowntree & Geraghty (1915) who used vital red. Dawson, Evans & Whipple (1920), who investigated a large number of dyestuffs with a view to their possible use in the determination of blood volume, showed that several dyes could be used for this purpose, but that a blue dye, T1824 or Evans Blue, was somewhat superior to all others. This dye combines with the plasma albumin to form a dye-protein complex (Rawson, 1943), and thus leaves the circulation relatively slowly (Gregersen & Rawson, 1943).

These two methods do not directly measure the total blood volume. The carbon monoxide method is a direct measure of the cell volume, while the dye method directly determines the plasma volume. In each case the figure for the total blood volume depends upon the assumption that the haematocrit value is the same in all parts of the circulation. If this assumption is correct, then both methods should give the same results for the total blood volume.

Several investigators have compared the values obtained by both methods with somewhat variable results. Smith, Arnold & Whipple (1921) made a detailed study in dogs, using brilliant vital red as the dye and the van Slyke & Salvesen (1919) method of estimating carbon monoxide in the blood. Their average results showed that the total blood volume by the carbon monoxide method was 86·9 c.c./kg. body weight and by the dye method 103·9 c.c./kg. They concluded that neither of these values was correct, but that the true blood volume was the sum of the red cell volume determined by the carbon monoxide method and the plasma volume determined by the dye. The discrepancies were postulated as being due to a lower haematocrit value in the arterioles and capillaries than in the larger blood vessels.

Bazett, Sunderman, Maxfield & Scott (1940) estimated the plasma volume with congo-red dye, and the cell volume by the carbon monoxide method in man. The true blood volume was taken as the sum of these two values. They found that the total blood volume calculated from the dye results was 103·2%, and that calculated by the CO results 96·2% of the true volume.

Asmussen (1941) determined the blood volume in man by both the carbon monoxide and dye (T1824) methods, and found that the results obtained by the carbon monoxide method were always greater than those given by dye. He concluded that this was due to the uptake of some of the carbon monoxide by myoglobin.

Hopper, Tabor & Winkler (1944) used both these methods simultaneously in man and dogs. They concluded that the results of both methods were often, but not always, numerically equivalent, but that average values were almost identical by the two methods.

Root, Roughton & Gregersen (1946) also determined the blood volume in man and dogs by both these methods. Their results by both methods are approximately the same.

The red cell volume has also been determined by measuring the dilution of injected red cells containing haemoglobin with radioactive iron in the molecule. Hahn, Ross, Bale, Balfour & Whipple (1942) determined the blood volume in dogs by this tagged red cell method and by the dye method (brilliant vital red). Their results showed that the red cell volume calculated from the plasma volume (dye) and jugular haematocrit was 10–40% higher than that estimated with the tagged red cells. Gibson, Peacock, Seligman & Sack (1946) have also measured the blood volume by the tagged red cell and dye (T1824) methods in both man and dogs. They also find that the red cell volume by the former method is much lower than by the dye method, the ratio of radio-iron red cell volume to dye red cell volume being 0·85 in humans and 0·82 in dogs. Meneely, Wells & Hahn (1947) also observe a similar discrepancy between these methods in man.

It is, therefore, evident that all investigators are not in agreement regarding the measurement of the blood volume by different methods. So further investigations have been made with the carbon monoxide method in man, and the blood volume has been determined in healthy subjects simultaneously by both this and the dye (T1824) methods.

METHODS

General procedure. The subjects in this investigation were all healthy young men—medical students, laboratory technicians and the authors. The body weight was measured with the subjects in the nude.

The carbon monoxide was administered into a closed circuit consisting of a Krogh spirometer, a soda-lime container and respiratory valves through which the subject breathed. The dead space was reduced to a minimum. Oxygen was admitted into the circuit to maintain the value of the spirometer nearly constant, and the carbon monoxide was always admitted on the inspiratory side of the valves. In an estimation of the blood volume by this method, about 2 l. of oxygen were introduced into the spirometer and the subject began breathing into the circuit. After 5 min. an initial blood sample was taken from an arm vein. The carbon monoxide was then introduced in 1–1½ min., after which samples of blood were taken at intervals. All samples were then stored in all-glass 1 c.c. syringes in the cold and dark after clotting had been prevented with dry heparin (Scholander & Roughton, 1943).

The carbon monoxide was made from formic acid and sulphuric acid and stored in an aspirating bottle over alkali (cp. Peters & van Slyke, 1932). It was made freshly every few days and always analysed before use and found to be 97·5–100 % pure. A known volume of carbon monoxide was introduced into the closed circuit apparatus by employing a gas-sampling tube with a 3-way tap at each end and surrounded by a water-jacket. By means of a mercury levelling bulb the sampling tube could be filled with carbon monoxide. The volume of the tube was 102·2 c.c., which usually corresponded to 90–95 c.c. pure CO at S.T.P.

For the measurement of the blood volume by the dye method, Evans Blue (T1824), made by Eastman Kodak (U.S.A.), was used. The dye was made up in watery solution in a concentration of 2·5 mg./c.c. Samples of this were autoclaved and the actual concentration of the autoclaved solution was checked by the photoelectric colorimeter against the solution not autoclaved to ensure that no change in strength had occurred. 5 c.c. of this solution, i.e. 12·5 mg. dye, were used for each determination.

In estimating the blood volume by the dye method, a blood sample was first taken from an arm vein. Then 5 c.c. of the dye solution was injected intravenously, and at 10, 20 and 30 min. after injection further blood samples were taken. In many instances, samples were taken at 5, 10, 20, 30, 45 and 60 min. after injection. The arm was always warmed in a water-bath at 44–45° C. and the blood samples withdrawn without stasis. Coagulation was prevented by dry heparin, and the dye concentration estimated in heparinized plasma. The

results were plotted and the curve extrapolated back to zero time to give the true plasma concentration.

Analytical methods

CO *in blood.* This was determined by the micro-gasometric method of Scholander & Roughton (1943), using the special method for small amounts of carbon monoxide in the blood. The analyses were all done in duplicate and the accuracy of the method in our hands was similar to that of Scholander & Roughton. Blood containing known concentrations of carbon monoxide was analysed with the results shown in Table 1.

TABLE 1. Accuracy of CO determinations in blood

Calculated (c.c./100 c.c.)	Determined (c.c./100 c.c.)	Calculated (c.c./100 c.c.)	Determined (c.c./100 c.c.)
0·90	0·95	2·13	2·11
1·67	1·62	2·41	2·42
1·73	1·77	2·49	2·48
1·97	2·01	2·49	2·52
2·00	1·99	3·00	3·09
2·00	2·02	3·24	3·25
2·11	2·09	4·40	4·39

CO *in the air in the lung-spirometer system.* The method used for this determination involved the absorption of the CO by human blood and the determination of the blood CO. It is a modification of methods used by many investigators.

A sample of air from the lung-spirometer system was taken into a 500 c.c. gas-sampling tube by the displacement of 30% NaCl solution. A small sample of air was also taken and analysed for CO_2 (always absent) and oxygen with the Haldane gas analysis apparatus. Into the original 500 c.c. sample, hydrosulphite solution (20% in N-NaOH plus 2% anthraquinone β sulphonate) was now run from a reservoir and the oxygen absorbed, leaving only nitrogen and CO. A tonometer of 70–100 c.c. volume (a gas-sampling tube was used) was now filled with this deoxygenated air and exactly 1 or 2 c.c. of human blood, deoxygenated by equilibration with pure nitrogen, was then introduced into the tonometer. This tonometer was then rotated in the dark at 16° C. for 2 hr. The CO content of the blood before and after rotation in the tonometer was then determined by the Scholander-Roughton method, the difference giving the uptake of CO by the blood. From this figure, the volume of the tonometer and the O_2% in the original air sample, the CO concentration in the original air can be calculated. Since there was practically no oxygen or carbon dioxide in the tonometer and the temperature only 16° C., the amount of CO remaining unabsorbed by the blood is very low, and has been neglected.

To test the accuracy of this method, air mixtures containing CO were made up in an accurately calibrated 11 l. aspirating bottle. The following results were obtained:

Actual % CO	Determined % CO
0·0207	0·0200, 0·0206, 0·0211, 0·0199—mean 0·0204
0·0206	0·0193, 0·0210, 0·0196, 0·0200—mean 0·0200.

This accuracy was sufficient for the determination of the residual CO in the lung-spirometer system, since the estimation of the volume of the system was much less accurate.

Haematocrit. An accurate and convenient method of determining the haematocrit value was found to be the following: a piece of glass tubing, having an external diameter of about 6 mm. and a bore of about 1·5 mm., was tested for uniformity of bore by introducing a drop of mercury and measuring its length at different points in the tube with the help of a travelling microscope. Any part of the tubing where the bore was not uniform was discarded and the rest cut into lengths 10 cm. long. To determine the haematocrit value, blood was sucked up into one of these 10 cm. tubes, and the lower end pressed firmly into a flat slab of plasticine to seal it off. This tube was then placed in a centrifuge cup and centrifuged for 1 hr. at 3000 r.p.m. The lengths of the columns of corpuscles and of corpuscles plus plasma were then read by means of a millimetre scale and the haematocrit value calculated. The advantage of using these tubes is that the method is simple and accurate, and the tubes can be washed out and used repeatedly. An analysis of variance on a series of forty-nine duplicate haematocrits shows that the standard error of the mean of a pair of duplicate determinations on the same sample of blood is 0·13%, the percentage here being the unit in which the haematocrit is measured.

Heparin was always used as anticoagulant, since potassium oxalate will cause shrinkage of the corpuscles as has been shown by other investigators. We have found that the haematocrit value of blood containing 0·1, 0·2 and 0·5% potassium oxalate is on the average 3·5, 6·7 and 15·2% respectively less than that of heparinized blood. When a mixture of two-thirds potassium and one-third ammonium oxalate is used, as recommended by Wintrobe, the difference is very much less, but errors may be produced by using 1% of this mixture (as employed by some authors), and these errors are likely to vary if the oxalate is not accurately measured. Therefore, if oxalate is used, the dye will be diluted in the plasma of the samples taken, so giving plasma volumes which are too high.

Haematocrit estimations were generally made on all samples of blood taken and the mean value used. In a series of twenty experiments samples were taken at 0, 10, 15 and 30 min. The mean haematocrit values of this series at these times were 42·8, 42·8, 42·8 and 43·1.

When examined statistically this series shows no regular change in haematocrit with time of sampling. The standard deviation of haematocrit in this series is 0·51%. The determinations are means of duplicates, so that the difference between this standard deviation and the standard error of the mean of duplicates given above must be ascribed to random variations from sample to sample of blood from the same individual.

It is now generally recognized that the haematocrit value, as determined, does not give a true measure of the percentage cell volume of the blood (Gregersen & Schiro, 1938; Shohl & Hunter, 1941), owing to some of the plasma being trapped between the corpuscles. In this investigation the determined

haematocrit value has been multiplied by the factor 0·96 to give the true haematocrit reading.

Dye in plasma. In all experiments in this investigation the dye in the plasma was first extracted with *n*-butyl alcohol according to the method of Harington, Pochin & Squire (1940) before the concentration was determined. 3 c.c. samples of plasma were used instead of 5 c.c. used by the original authors. This extraction in our hands has proved simple and accurate. It was found that the opalescence of the samples taken at 0, 10, 20 and 30 min. often varied considerably, especially after a fatty breakfast, thus leading to large errors if the dye was not extracted.

The apparatus used for the determination of the concentration of the dye consisted of a single photoelectric cell and moving coil mirror galvanometer. The light source was supplied by a 6 V. battery and could be adjusted in intensity by resistances in series. By means of a lens a parallel beam of light passed through the cell containing the butyl alcohol, 1 cm. in depth with a volume of 2·5 c.c., and a Wratten 29 filter, to the photocell.

To estimate the dye concentration in a sample the following procedure was adopted. With no light falling on the photocell, the reading on the scale was zero. The cell, *A*, was filled with the butyl alcohol from the undyed blood sample and placed in the apparatus. The light was now adjusted by means of the resistances so that after passing through the cell and falling on the photocell, the reading on the scale was 50 cm. (full scale). Cell *A* was now removed and another cell, *B* (filled with water), was placed in the apparatus and the reading observed. With cell *B* in the apparatus the light source was always adjusted if necessary to this reading immediately before cell *B* was removed and cell *A* introduced. The light source was thereby always kept constant and the intensity such as to give full scale deflexion with cell *A* in position and filled with butyl alcohol from undyed plasma. Adopting this procedure, the deflexion on the scale was determined when cell *A* was filled in turn with the butyl alcohol from the dyed plasma samples.

In the same way a calibration curve was made with plasma containing known concentrations of dye and extracted with butyl alcohol. This calibration curve was frequently checked and found not to alter, provided the apparatus was always kept in the same position. It was found that butyl alcohol extracts of normal undyed plasma from different individuals gave practically identical readings, so that by using a calibration curve made with plasma from another individual no significant error was involved. By this procedure, in repeated estimations of blood volume the original undyed plasma sample can always be used as the starting-point. This obviates the difficulties encountered when the first blood sample already contains dye. In none of the samples in our series was there any visible haemolysis, so errors due to haemolysed plasma must have been insignificant.

The accuracy of this method was tested with known concentrations of dye in human plasma The determined concentration was always within 3% of the actual concentration.

RESULTS

The absorption of CO *from the circuit.* The rate at which the CO was absorbed, and the amount of CO left behind in the circuit when equilibrium is reached, were determined in six experiments. The percentage of CO in the system at 5, 10 and 30 min. after the administration of the CO was estimated by the method described above. The results are given in Table 2. From this table it is evident that not quite all the CO is absorbed in 5 min., but that no further absorption takes place after 10 min.

The percentage of CO remaining in the system when equilibrium is reached will depend mainly on the percentage of oxygen present. The higher the oxygen, the higher will be the CO. In these experiments the oxygen percentage was kept unnecessarily high to make sure that there would be no oxygen lack.

In all experiments in this investigation, the same procedure was adopted, so that the percentage of oxygen should always have been approximately the same.

TABLE 2. Percentage of CO and O_2 in the lung-spirometer system at intervals after CO administration

	First administration (time in min.)						Second administration (time in min.)	
	5		10		30		10	
	CO	O_2	CO	O_2	CO	O_2	CO	O_2
R.W.G.	—	—	0·0219	48·2	0·0217	44·6	—	—
R.W.G.	0·0428	51·5	0·0245	49·6	—	—	0·0472	49·9
F.C.C.	—	—	0·0219	53·0	0·0208	50·8	0·0490	54·6
P.J.P.	0·0404	44·1	0·0218	44·0	0·0219	49·1	—	—
T.J.M.	—	—	—	—	0·0127	35·8	—	—
T.J.M.	—	—	0·0134	39·4	0·0131	49·4	0·0277	40·1

The actual volume of CO remaining in the lung-spirometer circuit can be calculated from the volume of the system and the percentage of CO. The volume of the system was estimated to be approximately 7000 c.c., but this would vary somewhat with the volume of reserve air in the lungs of different subjects, and with the amount of air in the spirometer, although this was kept as nearly constant as possible. The mean percentage of CO at 10 min. in five experiments was 0·0207. From these figures the average volume of CO remaining in the circuit when equilibrium is reached is approximately 1·4 c.c. or 1·6 % of the amount of CO administered.

In the calculation of the blood volume, therefore, this correction for unabsorbed CO has been made. When, at the end of 30 min., a further 102·2 c.c. CO is administered, the percentage of CO left in the circuit after 10 min. rebreathing is approximately doubled. Thus about the same percentage of the second dose of CO remains unabsorbed, so that the same correction has to be made.

Initial CO *in blood.* As pointed out by several investigators, it is important to determine the initial blood CO content even in non-smokers. Courtice & Simmonds (1948) give figures to show how greatly this varies especially in cigarette smokers.

The blood CO *at intervals after administration of* CO *during rest.* The CO content of the blood was determined before and at 5, 10, 15, 30, 45 and 60 min. after the administration of CO in eleven experiments on seven subjects. The subjects breathed through the circuit continuously for this time. The average blood CO in c.c. per cent for these eleven experiments was 0·32 before and 2·00, 2·00, 1·96, 1·91, 1·88 and 1·87 at 5, 10, 15, 30, 45 and 60 min. after CO administration. The curve in every individual case rose to a maximum in 5 or 10 min., and then showed a very gradual downward trend, so the average figures give a true picture of the behaviour of the blood CO during this time.

In a larger series of experiments, twenty-five in all, the blood CO content has been determined before and at, 5, 10, 15 and 30 min. after CO administration. In all cases the maximum concentration is observed at 5 or 10 min. and then there is a very gradual fall. Individual figures are given in Table 3. It

TABLE 3. The blood carbon monoxide, c.c./100 c.c., before and after administration of carbon monoxide in human subjects at rest

		Time after administration (min.)			
	Before	5	10	15	30
	0·20	2·12	2·05	1·99	1·97
	0·22	2·04	2·05	1·99	1·93
	0·24	2·13	2·08	2·05	1·99
	0·17	2·08	2·11	2·08	2·06
	0·57	2·17	2·14	2·02	1·91
	0·57	2·13	2·12	2·07	1·98
	0·53	2·05	1·99	1·99	1·99
	0·80	2·45	2·36	2·33	2·32
	0·65	2·30	2·25	2·19	2·20
	0·28	1·75	1·75	1·75	1·75
	0·17	1·85	1·91	1·89	1·85
	0·14	2·11	2·08	2·03	1·93
	0·12	1·74	1·79	1·74	1·67
	0·27	1·97	2·03	2·03	2·00
	0·34	2·32	2·29	2·22	2·16
	1·01	2·57	2·55	2·51	2·48
	0·17	1·79	1·74	1·67	1·66
	0·17	1·82	1·81	1·82	1·76
	0·65	2·21	2·23	2·12	2·00
	0·47	1·92	2·09	2·00	2·00
	1·96	3·87	3·89	3·89	3·84
	2·17	3·81	3·75	3·73	3·70
	2·09	3·87	3·90	3·83	3·83
	1·81	3·60	3·55	3·55	3·58
	1·55	3·12	3·08	3·03	3·03
Mean	0·69	2·39	2·38	2·34	2·30

has been seen that almost all the CO has been absorbed by 5 min. If it be assumed that mixing is complete by 10 min., it is evident that there is a slow disappearance of CO from the blood from 10 to 30 min. amounting to 0·08 volume per cent on the average, or $\frac{0{\cdot}08}{2{\cdot}38-0{\cdot}69}\times 100\,\% = 4{\cdot}7\,\%$ of the added CO.

This fall cannot be accounted for by a dilution of the blood during the time of the experiment. The average haematocrit values of blood samples taken at 0, 10, 15 and 30 min. in twenty experiments were 42·8, 42·8, 42·8 and 43·1 respectively. The amount of CO taken up in solution by the body fluids is insignificant, amounting to only 0·2 % of the CO administered, at a tension of 0·16 mm. (0·021 % CO), assuming that 60 % of the body is water. Thus this gradual disappearance of CO appears to be due to CO combining chemically with some substance outside the normally circulating blood.

Blood CO *after administration of* CO *during exercise.* The most likely substances outside the circulation are myoglobin and non-circulating red cells.

Experiments with radioactive iron indicate that there are very few non-circulating red cells (Hahn *et al.* 1942). If CO, at the low tension present in the blood, slowly diffuses into the tissue fluids and is therefore slowly taken up by the myoglobin, then it is possible that this slow fall is due to the formation of CO-myoglobin. If this is so, the theoretical amount of CO in the blood at zero time could be obtained by extrapolating and so allowing for this disappearance.

If this is the case, during moderate muscular exercise when the circulation to the muscles is considerably increased, the fall in blood CO should be more rapid. Carbon monoxide was, therefore, administered during muscular exercise and blood samples taken before and 5, 10, 15 and 30 min. after CO. The subject pedalled a Krogh electric-brake ergometer for 15 min. before administration of the CO and continued pedalling for 30 min. afterwards, during which time he breathed through the circuit. The load was moderate (420 kg.m./min.), but was increased slightly in some subjects. The seve'ty of the exercise is evident from the oxygen consumption determined by the Douglas bag technique.

TABLE 4. The blood carbon monoxide, c.c./100 c.c., before and after administration of carbon monoxide in human subjects during moderate exercise on the bicycle ergometer

		Blood CO				
			Time after CO administration (min.)			
Work done (kg.m./min.)	Oxygen consumption (c.c./min.)	Before	5	10	15	30
420	1072	0·15	1·97	1·98	1·91	1·95
420	1333	0·54	2·16	2·10	2·05	2·08
630	1604	0·56	2·60	2·63	2·58	2·59
525	1439	0·05	1·48	1·54	1·49	1·53
420	1144	1·86	3·85	3·87	3·85	3 81
420	1126	2·47	4·09	4·03	3·99	3 97
420	1167	1·93	3·57	3·54	3·48	3·48
420	1119	1·60	3·29	3·22	3·23	3·26
	Mean	1·15	2·87	2·86	2·82	2·83
Mean haematocrit × 0·96		42·9	—	43·1	43·4	43·4

The results of eight experiments are given in Table 4. In four of these experiments the initial CO was low and in the other four high, the experiments in this latter group being done immediately after a resting blood volume determination had been made. The average fall in blood CO in these eight experiments is the same from 5 to 10 and from 10 to 15 min. as in the resting group, but from 15 to 30 min. there is no further fall. The group is not large enough to say definitely whether this is significant or not, as the differences are so small and come within the errors of the analytical method. These results, however, suggest that there may be a more rapid uptake of CO earlier, and that equilibrium with the myoglobin is reached more quickly, but that the actual disappearance of CO from the blood is small in either case from 5 min. onwards. That there can be no large uptake of CO before 5 min. in exercise which did not occur at rest is evident from the values obtained for the blood volume at

rest and during exercise immediately afterwards (Table 6), and also from the values obtained during exercise not preceded by a resting determination (Table 7). The effect of the exercise used here on the cardiac output was determined in three subjects by Grollman's acetylene method. The results given in Table 5 show that the cardiac output is approximately trebled, and the blood supply to the active muscles will be even more greatly increased. The active muscles, however, represent only a portion of the whole.

TABLE 5. The cardiac output and pulmonary ventilation during exercise, 420 kg.m./min. on bicycle ergometer

Subject		Pulm. vent. (l./min. S.T.P.)	O_2 consumption (c.c./min.)	Pulse rate/min.	Cardiac output (l./min.)
F.C.C.	Rest	5·43	261	74	4·2
	Exercise	17·81	1127	104	12·2
R.W.G.	Rest	6·92	350	68	6·2
	Exercise	21·10	1376	106	18·8
T.J.M.	Rest	6·42	341	73	4·6
	Exercise	23·40	1272	90	16·9

Repeated determinations of blood volume by CO *method.* It is possible that in addition to this slow loss of CO from the blood there is an extremely rapid uptake by the myoglobin before the first sample is taken at 5 min. whether the subject is at rest or exercising. To find whether any difference would be observed if a second administration were made, the blood CO was followed for 30 min. after the first administration when a second dose was given and the blood CO level followed for a further 30 min. This was done with the subject at rest all the time, and also with the subject at rest for the first administration and exercising on the ergometer during the second. The results of these experiments are given in Table 6. The blood volume is practically the same in repeated estimations whether these be made twice at rest, or at rest and during exercise.

TABLE 6. The blood volume in c.c. by carbon monoxide method repeated 20 min. after first estimation

	Blood volume 1				Blood volume 2			
Subject	Haematocrit ×0·96	Cells	Plasma	Total	Haematocrit ×0·96	Cells	Plasma	Total
		Rest				Rest		
F.C.C.	40·0	1880	2830	4710	40·2	1910	2830	4740
P.J.P.	40·8	2040	2950	4990	39·3	2000	3080	5080
R.W.G.	43·3	2460	3230	5690	42·7	2420	3240	5660
T.J.M.	40·3	2320	3430	5750	40·4	2380	3500	5880
Mean	41·1	2180	3110	5290	40·7	2180	3160	5340
		Rest				Exercise		
F.C.C.	41·4	1910	2700	4610	41·2	1870	2680	4550
P.J.P.	41·8	2230	3100	5330	42·0	2200	3030	5230
R.W.G.	44·0	2570	3280	5850	44·5	2520	3150	5670
T.J.M.	43·1	2380	3140	5520	43·1	2370	3140	5510
Mean	42·6	2270	3060	5330	42·7	2240	3000	5240

Thus, if myoglobin takes up a considerable quantity of CO rapidly before 5 min., it seems obvious that it must take up an extra identical amount when a second administration of CO is given whether the subject be at rest or exercising.

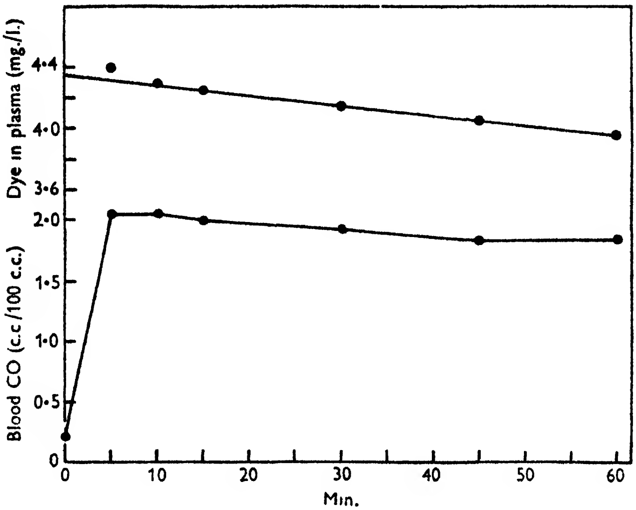


Fig. 1. The concentrations of CO in the blood and of dye in the plasma at intervals after the administration of carbon monoxide and intravenous injection of dye.

TABLE 7. The blood volume determined simultaneously by carbon monoxide and dye methods

Subject	Age	Wt. (kg.)	Haema-tocrit actual ×0·96	Blood volume by carbon monoxide (c.c.) Cells	Plasma	Total	Blood volume by dye (c.c.) Cells	Plasma	Total	CO blood volume / Dye blood volume
						Rest				
F.C.C.	36	66·1	40·6	1980	2900	4880	1870	2740	4610	1·06
F.C.C.	36	66·6	40·9	1910	2760	4670	1830	2650	4480	1·04
R.W.G.	24	82·2	43·4	2650	3460	6110	2600	3390	5990	1·02
R.W.G.	24	80·7	44·0	2400	3070	5470	2480	3150	5630	0·97
R.W.G.	24	80·7	44·0	2620	3330	5950	2590	3290	5880	1·01
T.J.M.	30	86·8	43·4	2620	3420	6040	2520	3290	5810	1·04
M.V.B.	20	64·8	40·5	1960	2840	4800	2000	2940	4940	0·97
G.R.	21	59·2	46·1	1970	2310	4280	1970	2300	4270	1·00
W.M.J.	20	62·3	43·1	2150	2840	4990	1950	2580	4530	1·10
P.J.R.P.	20	64·2	45·7	2390	2840	5230	2330	2770	5100	1·03
C.G.M.	20	67·7	44·2	2280	2880	5160	2130	2690	4820	1·07
W.W.W.	25	88·2	42·8	2490	3330	5820	2560	3420	5980	0·97
R.G.T.	20	70·6	47·2	2560	2870	5430	2600	2910	5510	0·99
H.	21	63·6	45·1	2120	2570	4690	1960	2390	4350	1·08
J.M.L.G.	22	60·5	47·2	2420	2710	5130	2230	2490	4720	1·09
J.M.L.G.	22	60·5	47·4	2310	2580	4890	2230	2480	4710	1·04
									Mean	1·03
						Exercise				
F.C.C.	36	66·4	40·8	1960	2840	4800	1880	2720	4600	1·04
R.W.G.	24	81·5	47·1	2570	2890	5460	2500	2810	5310	1·03
W.J.S.	28	61·4	45·5	1980	2380	4360	1810	2170	3980	1·10
T.J.M.	30	86·8	44·3	2460	3090	5550	2470	3100	5570	1·00
									Mean	1·04

In calculating the blood volume by the CO method, the blood CO at 10 min. has been taken and 1·6 % of the administered CO deducted. No correction has been made to allow for the slow disappearance of CO from 10 to 30 min., which on the average has been shown to be 4·7 % of the administered CO during this time.

Simultaneous determination of blood volume by CO *and dye methods.* The blood volume has been determined simultaneously by both methods in twenty experiments. The dye was injected at exactly the same time as the CO was administered. A typical experiment is depicted in Fig. 1, and individual results are given in Table 7.

These figures indicate that the blood volume is very nearly the same by both methods, but, on the average, the blood volume is about 3 % higher by the CO than by the dye method. This is so when no correction is made for the slow disappearance of CO which is evident from 10 to 30 min. On the average about 5 % of the administered CO disappears from the blood in these 20 min. If then the CO is disappearing at about the same rate during the first 10 min., the blood volume by the CO method should be reduced by about 2–3 %, so that there will then be no significant difference between the results obtained by both methods.

DISCUSSION

Although the principle of the dye estimation of plasma volume is simple, the actual determination of the dye concentration in the plasma when mixing is complete and no dye has left the circulation is by no means straightforward. The mixing time and rate of disappearance of the dye from the plasma have led to much controversy, and the procedure adopted by different investigators varies. Besides this, the actual measurement of dye in the samples of plasma or serum may lead to errors if the opalescence of the serial samples differs, as it often does. In our experience this opalescence may cause large errors if the subject is not in the postabsorptive state. For this reason we used the Harington, Pochin & Squire technique for extracting the dye with *n*-butyl alcohol. We do not find this procedure laborious, and in our hands it gives reliable and accurate results when plasma samples containing known concentrations of dye are used.

The time for complete mixing and the disappearance curve have been discussed by many investigators and will not be further discussed here. We have taken blood samples at 5, 10, 20, 30, 45 and 60 min. in several cases and at 10, 20 and 30 min. in others, plotted the results and extrapolated to zero time as shown in Fig. 1.

Gibson, Peacock, Seligman & Sack (1946) state that modern modifications employing T1824 have clearly shown that plasma volume can be measured with a high degree of accuracy in normal men and animals and that values for plasma volume in man determined by several workers are in general agreement

both as to absolute plasma value and plasma volume per unit of body measurement.

TABLE 8. The plasma volume, c.c./kg., and the mean square, of normal male subjects determined by the dye method

Author	Dye used	Number of subjects	Plasma volume (c.c./kg.)	Mean square
Courtice & Gunton	T1824	12	40·5	4·85
Davis (1942)	T1824	11	40·5	32·10
Gibson & Evans (1937)	T1824	49	43·1	34·66
Noble & Gregersen (1946)	T1824	51	44·7	—
Stewart & Rourke (1941)	T1824	11	45·6	42·05
Hopper, Tabor & Winkler (1944)	T1824	5	46·4	29·87
Harington, Pochin & Squire (1940)	T1824	11	47·7	33·83
Gibson, Peacock, Seligman & Sack (1946)	T1824	40	48·0	29·78
Root, Roughton & Gregersen (1946)	T1824	11	48·6	52·13
Crooke & Morris (1942)	T1824	5	49·8	—
Mather, Bowler, Crooke & Morris (1947)	T1824	53	54·8	49·15
Sunderman & Austin (1936)	Vital red	9	43·2	2·39
Keith, Rowntree & Geraghty (1915)	Vital red	42	48·9	10·49

This statement should be further considered. In Table 8 the values for plasma volume, determined by different investigators on healthy male subjects at rest, are given. These are expressed as the mean plasma volume in c.c. per kg. and the mean square has been calculated for each series to give a measure of the magnitude of tendency to variation in the results. It can be seen that the mean plasma volume varies considerably in the different groups. Our values give a low mean and a low variance, but are not statistically different from those of the larger series of Gibson & Evans (1937). The values for plasma volume obtained by Gibson, Peacock, Seligman & Sack (1946) are about 19% higher than our results and 11·5% higher than those of Gibson & Evans, and both these differences are statistically significant, thus representing a real difference. The values obtained by Mather, Bowler, Crooke & Morris (1947) are very much greater than ours (35%). It is possible that the true plasma volume of one group of men may be different from that of another depending on the season and environmental temperature. It is, however, also probable that the discrepancies between all the results in Table 8 are due to differences in procedure for the estimation of dye dilution. In some cases dye concentration in single samples at 4 or 10 min. after injection have been used. In others, the values at 20, 40 and 60 min. have been averaged. Some workers have plotted the dye concentrations at 30, 60 and 90 min. and extrapolated the curve to zero time, while others have plotted the values at 10, 20 and 30 min. linearly or semi-logarithmically and extrapolated to zero time.

There is in fact no general agreement in procedure and values obtained for plasma volume determinations in man using the dye method. Yet in investigations designed to compare venous and body haematocrit it has been assumed that the dye method employed gives a true unequivocal value for plasma volume.

The discrepancies in plasma volume observed in Table 8, therefore, indicate that the accuracy of this value by the dye method is difficult to determine, and may in part account for differences observed by various investigators when estimating the blood volume by two methods.

Root *et al.* (1946), and Roughton & Root (1946) have fully discussed the carbon monoxide method for blood volume and the disappearance of carbon monoxide from the blood. Their arguments apply to our work and need not be repeated. From our results, as from theirs, a slow diffusion of CO from the blood is apparent, but it is impossible to say dogmatically whether or not there is a large uptake of CO by the myoglobin in the first 5 min. However, since the cardiac output per minute at rest is approximately equal to the blood volume, and since only about one-fifth of the cardiac output probably goes to the resting muscles, it seems unlikely that any large fraction of the carbon monoxide diffuses into the myoglobin in the first few minutes, especially as the affinity of myoglobin for carbon monoxide compared with oxygen is only a twentieth of that of blood haemoglobin. It is more probable, therefore, that the diffusion into the myoglobin will be a slower process, and that the fall we have noted represents this diffusion. If this is so, we are justified in correcting for this factor. In exercise, when the blood flow to the active muscles is increased, there may be a more rapid diffusion to the active muscles followed by a slow diffusion to the inactive muscles. What we have found is that the fall after 10 min. in exercise is, if anything, smaller than at rest, but that there is no significant difference in blood volume at rest and exercise. It is thus probable that at rest the slow fall is the only uptake of CO by myoglobin, whereas in exercise part of this fall is masked by a more rapid uptake of CO by the active muscles followed by a slower fall due to the uptake by the inactive muscles. It would appear, therefore, that the total uptake of carbon monoxide is at any rate not very great either at rest or during exercise, but there is no conclusive evidence for this.

The fact that the blood volume in our results is about the same by both the carbon monoxide and dye methods does not necessarily mean that the haematocrit value is the same throughout the circulation. Gibson, Seligman, Peacock, Aub, Fine & Evans (1946) give evidence to show that the blood in the small vessels of dogs under light morphine anaesthesia is only 17 % of the total blood volume. Thus there would have to be large changes in haematocrit in the small vessels to account for the discrepancies in the carbon monoxide or radio-iron and dye methods which have been observed by some authors.

Whether our results are correct or not can best be determined by measuring the red cell volume by the carbon monoxide and radio-iron techniques simultaneously. By the procedures we have adopted for the determination of the blood volume by the carbon monoxide and dye methods in normal healthy man, there is no significant difference between the results obtained by

both methods. The carbon monoxide method has certain advantages over the dye method. Small blood samples only are needed, opalescence and haemolysis in a sample do not affect the results, and repeated determinations can be made at fairly short intervals over an indefinite time, whereas repeated dye determinations will eventually give the subject a greenish blue tint.

SUMMARY

The blood volume has been determined in healthy young men by the carbon monoxide and dye (T 1824) methods simultaneously.

By the procedure adopted, there is no significant difference between the values obtained by both methods.

Sources of error by both methods have been discussed.

We are indebted to Dr R. B. Fisher for statistical analyses of results and to Messrs P. J. Phipps and T. J. Meadows for technical assistance.

REFERENCES

Asmussen, E. (1941). *Act. Physiol. Scand.* **3**, 156.

Bazett, H. C., Sunderman, F. W., Maxfield, M. E. & Scott, J. C. (1940). *Amer. J. Physiol.* **129**, 309 *P*.

Courtice, F. C. & Simmonds, W. J. (1948). *J. Physiol.* **107**, 300.

Crooke, A. C. & Morris, C. J. O. R. (1942). *J. Physiol.* **101**, 217.

Davis, L. J. (1942). *Edinb. med. J.* **49**, 465.

Dawson, A. B., Evans, H. M. & Whipple, G. H. (1920). *Amer. J. Physiol.* **51**, 232.

Gibson, J. G. & Evans, W. A. (1937). *J. clin. Invest.* **16**, 317.

Gibson, J. G., Peacock, W. C., Seligman, A. M. & Sack, T. (1946). *J. clin. Invest.* **25**, 838.

Gibson, J. G., Seligman, A. M., Peacock, W. C., Aub, J. C., Fine, J. & Evans, R. D. (1946). *J. clin. Invest.* **25**, 848.

Gregersen, M. I. & Rawson, R. A. (1943). *Amer. J. Physiol.* **138**, 698.

Gregersen, M. I. & Schiro, H. (1938). *Amer. J. Physiol.* **121**, 284.

Hahn, P. F., Ross, J. F., Bale, W. F., Balfour, W. M. & Whipple, G. H. (1942). *J. exp. Med.* **75**, 221.

Haldane, J. S. & Smith, J. L. (1900). *J. Physiol.* **25**, 331.

Harington, C. R., Pochin, E. E. & Squire, J. R. (1940). *Clin. Sci.* **4**, 311.

Hopper, J., Tabor, H. & Winkler, A. W. (1944). *J. clin. Invest.* **23**, 628.

Keith, N. M., Rowntree, L. G. & Geraghty, J. T. (1915). *Arch. intern. Med.* **16**, 547.

Mather, K., Bowler, R. G., Crooke, A. C. & Morris, C. J. O. R. (1947). *Brit. J. exp. Path.* **28**, 12.

Meneely, G. R., Wells, E. B. & Hahn, P. F. (1947). *Amer. J. Physiol.* **148**, 531.

Noble, R. P. & Gregersen, M. I. (1946). *J. clin. Invest.* **25**, 172.

Peters, J. P. & van Slyke, D. D. (1932). *Quantitative Clinical Chemistry.* Vol. II, *Methods.*

Rawson, R. A. (1943). *Amer. J. Physiol.* **138**, 708.

Root, W. S., Roughton, F. J. W. & Gregersen, M. I. (1946). *Amer. J. Physiol.* **146**, 739.

Roughton, F. J. W. & Root, W. S. (1946). *Amer. J. Physiol.* **145**, 239.

Scholander, P. F. & Roughton, F. J. W. (1943). *J. biol. Chem.* **148**, 551.

Shohl, A. T. & Hunter, T. H. (1941). *J. Lab. clin. Med.* **26**, 1829.

Smith, H. P., Arnold, H. R. & Whipple, G. H. (1921). *Amer. J. Physiol.* **56**, 336.

Stewart, J. D. & Rourke, G. M. (1941). *J. Lab. clin. Med.* **26**, 1383.

Sunderman, F. W. & Austin, J. H. (1936). *Amer. J. Physiol.* **117**, 474.

van Slyke, D. D. & Salvesen, H. A. (1919). *J. biol. Chem.* **40**, 103.

J. Physiol. (1949) 108, 157–159 616–073.173:612.179.2

A FOETAL PLETHYSMOGRAPH

By A. D. M. GREENFIELD

From the Physiology Department, St Mary's Hospital Medical School, London

(*Received* 3 *March* 1948)

Observation of changes of foetal volume provides a method for studying a number of problems in foetal physiology. Among these are fluctuations in the partition of the foetal blood between foetus and placenta, the rate of blood flow in the umbilical cord, and rapid fluid exchanges between foetus and mother.

The desirable features of a foetal plethysmograph are as follows:

(1) The seal between the umbilical cord and the plethysmograph must be made without pressing on, and preferably without touching, the cord, on account of the extreme irritability of the vessels within it.

(2) It should be easy and quick to apply.

(3) The sealing of the plethysmograph should be immediate and positive.

(4) The foetus and the umbilical cord should be totally immersed in physiological saline at body temperature, to simulate conditions in utero.

(5) The foetus should be clearly visible within the plethysmograph, so that any movements may be noted.

Plethysmographs have been designed and made which fulfil all these criteria with the exception that a short length of the umbilical cord passes out of the saline bath and lies on a rounded Perspex strip.

Four plethysmographs have been used. The largest has been successfully employed with sheep foetuses of 1700–4200 g. weight (Fig. 1). It is basically an open-topped tank of 14-gauge tinned brass, 38 cm. long, 20 cm. wide and 14 cm. deep. One of the lower long edges is bevelled, so that on this side the vertical wall is only 6 cm. deep. In use, this bevel fits against the lower abdomen of the mother and allows the plethysmograph to be brought more closely to her uterus exposed through a laparotomy opening and brought out on to the abdominal wall.

The rim of the plethysmograph is provided with a 3 cm. wide out-turned flange, and care is taken that the upper surface is smooth and flat. Where the walls meet the flange the edge is rounded off with a radius of about 0·5 cm. The umbilical cord passes over the brim at a distance of 13 cm. from one end. Soft rubber draught excluder is arranged round the top surface of the flange except at the place where the cord is to enter. Here, the ends of the rubber are carried vertically downwards for 5 cm. at a distance of 4 cm. apart on the inside of the wall.

To provide a smooth support for the cord a Perspex strip, 2·5 cm. wide within the plethysmograph and 6 cm. wide outside it, is bent over and attached to the flange as shown in Fig. 2. Small electric bulbs are arranged below this strip to transilluminate the cord, and holes are drilled in the flange to allow light to pass.

Recesses for the fore and hindlimbs, 6 cm. deep and 5 cm. square, are made in the side of the plethysmograph, one on each side of the entry point for the umbilical cord. The tops of the recesses are 1 cm. below the brim, so that the flange is left clear to take the clamps for fixing the lid. The foot recesses allow the umbilicus to be brought close to the entry point of the cord.

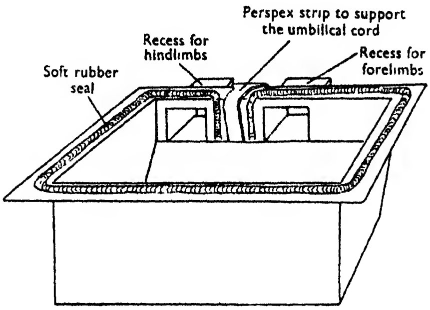


Fig. 1. Perspective drawing of the plethysmograph tank.

The lid of the plethysmograph (Fig. 3) is made of ¼ in. Perspex sheet. A notch, 2·8 cm. wide and 4 cm. deep, as measured from the wall of the plethysmograph, is cut in one side to allow the cord to enter. A curtain of Perspex 3 cm. deep hangs from the edges of the notch and the ends of the curtain are turned outwards to make shoulders which press against the vertical parts of the rubber

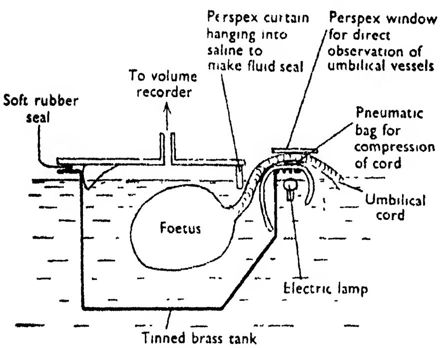


Fig. 2.

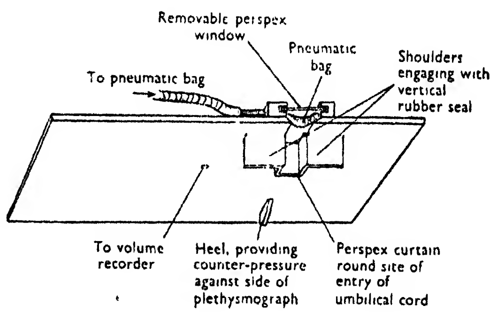


Fig. 3.

Fig. 2. Section through the plethysmograph at the point of entry of the umbilical cord. The umbilical cord is seen in position, lying on a gently curved Perspex strip.

Fig. 3. Perspective drawing of the lid of the plethysmograph, as seen if held in the normal horizontal position, and viewed from below.

seal. A heel of Perspex on the under-side of the lid provides counter-pressure against the opposite side of the plethysmograph, and holds the vertical joint tight. Where the shoulders meet the lid the angle is filled in with Perspex, shaped to a smooth curve of 1 cm. radius. The lid is held in position by eight clamps which effect a tight seal. The lid is provided with a tube connecting with the volume recorder, and a 1 cm. hole for use in calibrating, which is normally closed by a vaselined Perspex plate.

Provision for compression of the cord is made at the point where the cord passes over the brim. A rubber finger stall, which can be inflated as desired from a reservoir, is arranged to lie transversely across the path of the cord, and a removable Perspex window is arranged over it so that

there is a gap of 1·5 cm. when the finger stall is deflated. The finger stall is of sufficient size to fill the gap completely when it is distended at minimal pressures. Both finger stall and window are attached to the lid. Provision is made to hold the plethysmograph rigidly in position when in use, as any movement during an observation is inadmissible.

The following sizes of plethysmograph have been found suitable at different foetal ages. In each case the last figure is the depth. The cord enters at a point one-third along a long side. All have limb recesses:

Age (days)	Weight (g.)	Plethysmograph dimensions (cm.)
60	50– 100	12 × 6 × 4
70–100	100– 750	20 × 10 × 7
100–120	750–1600	28 × 13 × 10
120–143	1700–4200	38 × 20 × 14

Principles in use. The effective working of the plethysmograph depends on the fluid seal provided when it is filled to a depth sufficient to immerse the lower edge of the Perspex curtain round the point of entry of the cord. In practice it is filled to within 1 cm. of the brim, and it is immersed as a whole in a bath to the same level.

It will operate only in conjunction with a volume recorder which is so balanced that it exerts no back pressure in any static position, and a minimum of back pressure due to dynamic factors. In these conditions the level of the fluid in the plethysmograph remains always the same as that in the trap through which the cord enters. A change in volume of the foetus therefore alters the level in the two compartments equally, and the volume displaced in each compartment is proportional to the fluid area exposed in it. For this reason, the compartment through which the cord passes is made as small as possible, compatible with complete freedom of the cord, and the fluid surface area within it is very small indeed when the cord is in place. The smaller this area is made, the greater is the tolerance of dynamic imperfection in the volume recorder.

It follows from this that the volume of air displaced from under the lid of the plethysmograph is a definite and very large fraction of any volume changes in the foetus. The precise size of the fraction need not be known, as the plethysmograph and volume recorder are calibrated as a whole with the foetus and cord in position, or a suitable rubber tube may be used to represent the cord.

SUMMARY

A foetal plethysmograph is described which avoids the use of a contact seal at the point of entry of the umbilical cord.

I wish to thank Dr C. J. Hodson for a remark which led me to the design of the seal for the umbilical cord.

J. Physiol. (1949) 108, 160–166 612.179.2

THE UMBILICAL BLOOD FLOW IN THE FOETAL SHEEP

By K. E. COOPER, A. D. M. GREENFIELD and A. St G. HUGGETT

From the Physiology Department, St Mary's Hospital Medical School, London

(*Received* 3 *March* 1948)

The rate of umbilical blood flow has been measured in sheep foetuses between the 60th and the 143rd day of pregnancy.

METHOD

The foetuses were delivered under saline at 37° C. by the technique of Huggett (1927) from Welsh ewes of 30–45 kg. weight, under spinal anaesthesia. Blood flows were measured by the method of Cooper & Greenfield (1948) using the foetal plethysmograph (Greenfield, 1948). In most cases observations commenced within a few minutes of opening the uterus.

RESULTS

The accuracy of the measurements naturally increased as experience was gained in handling the animals and apparatus, and as improvements were effected in the design and construction of the plethysmograph and recording gear. In order to assess the value of the data in this paper, Table 1 gives a list of the ewes observed in chronological order. The foetal condition, as judged by its activity and muscle tone, and particularly the pressures required to collapse the vessels in the umbilical cord, is stated in each case. The foetus was considered to be in good condition if the umbilical arteries were unaffected by a bag pressure of 25 mm. Hg in a 60-day foetus, 35 mm. Hg at 100 days of age, and 50 mm. Hg at 130 days of foetal age. In the first five experiments (up to and including ewe no. 126), however, the umbilical veins were obstructed by raising a hydrostatic pressure in the bag until the column of blood in the veins was interrupted. No measurements of the pressure were made, and knowledge of the state of the foetal circulation is less certain in these cases.

Table 1 also shows the extreme limits of inaccuracy in the recording apparatus used on each occasion, as shown by inconsistencies revealed in the overall dynamic calibration (Cooper & Greenfield, 1948) of the various combinations of plethysmograph and volume recorder. All experiments are presented because the limited supply of material available does not permit repetition of all the earlier and less satisfactory measurements.

TABLE 1. The chronological order of the experiments with an estimate of the probable accuracy of the measurements, and the condition of the foetus

Date	Ewe no.	Foetal age (days)	Foetal weight (g.)	Foetal condition: Movements and muscle tone	Foetal condition: Bag pressure just failing to diminish flow in umbilical arteries (mm. Hg)	Estimate of possible error in recording (%)
31. i. 47	154 T	100	630		?	±15
5. ii. 47	164	105	780		?	±15
14. ii. 47	141	115	1722		?	±15
20. ii. 47	165	121	1791		?	±15
21. ii. 47	126	125	2109		?	±15
26. ii. 47	162	130	2179		40	±10
28. ii. 47	151 T	134	2120		50	±10
19. iii. 47	194	143	4200		?	± 5
21. iii. 47	136	130	2600		50	±10
28. iii. 47	183 T	143	2480	Poor	35	± 5
2. iv. 47	168	140	3320	Very good	50	± 5
9. i. 48	217	71	109	Excellent	30	± 5
13. i. 48	234	62	68	Excellent	25	± 5
16. i. 48	202	80	255	Excellent	35	± 3
20. i. 48	205 T	100	881	Very good	35	± 5
21. i. 48	218 T	90	398	Fair	25	± 5
	218 T	90	464	Very good	35	± 5
30. i. 48	220	110	1180	Very good	40	± 5
4. ii. 48	226	116	1347	Very good	40	± 5
11. ii. 48	210	105	917	Very good	30	± 5
25. ii. 48.	231	119	1643	Fair	30	± 5

T indicates one of twins.

TABLE 2. Umbilical blood flows arranged in order of foetal age

Ewe no.	Foetal age (days)	Average blood flow (ml./min.)	Range of blood flow (ml./min.)	No. of observations averaged	Average blood flow/kg. foetal wt. (ml./min.)
234	62	19	13–25	10	279
217	71	19	16–22	4	174
202	80	53	48–59	4	210
218 T	90	61	54–69	4	153*
218 T	90	85	52–108	5	186
205 T	100	194	175–217	5	220
154 T	100	85	60–126	9	135
164	105	140	138–145	4	188
210	105	171	158–180	10	185
220	110	226	170–280	10	194
141	115	268	209–303	10	156
226	116	310	280–330	10	230
231	119	233	220–265	10	151
165	121	219	192–260	10	123
126	125	271	247–305	10	128
162	130	260	245–282	6	120
136	130	245	231–262	5	95*
151 T	134	352	310–400	8	166
168	140	522	500–555	10	162
194	143	437	335–490	6	104
183 T	143	209	165–262	10	84*

T indicates that the foetus was one of twins.
In the case of no. 183, the second twin was dead and undergoing maceration.
* Indicates that the foetus was in poor condition.

Relationship of umbilical blood flow to foetal age. The umbilical blood flows of the series are shown in Table 2. The observations reported in this table were the first few made on each foetus and, apart from ewe no. 136, there had been no interference with the cord. In ewe no. 136, a needle was inserted into one umbilical artery for pressure measurements, and the observations were commenced 46 min. after opening the uterus. Most of the other observations were made within a few minutes of removal of the foetus from the uterus. If no. 154, the first and least satisfactory animal, is excluded, 111 observations are

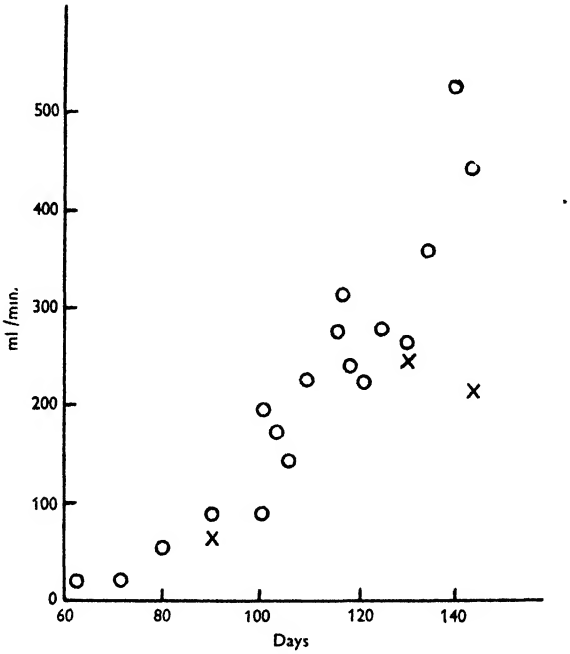


Fig. 1. Umbilical blood flow in sheep at different foetal ages. Ordinate: umbilical blood flow. Abscissa: foetal age. Each circle represents the average of between four and ten observations on a single foetus. Crosses represent results obtained on foetuses in poor condition.

reported in the table. Of these, thirty-four observations differ from the average for the same foetus by more than 10 %, and ten differ from the average by more than 20 %. These fluctuations in observed blood flow appear to be, at least in part, a real phenomenon, because they were as great in the 1948 series as in the 1947, although the former were technically much more satisfactory. The average blood flow for each foetus is plotted against the foetal age in Fig. 1.

Relationship of umbilical blood flow to foetal weight. The average blood flow per kg. foetal weight for each foetus is shown in Table 2 and in Fig. 3. It will be observed that there is, on the whole, a decline from about 250 ml./kg./min. at the earlier ages, to about 130 ml./kg./min. at the later ages.

Relationship of umbilical blood flow to the rate of foetal growth. The relationship of foetal weight to foetal age for all foetuses on which the umbilical blood flow has been measured, and a number of other foetuses of the same breed and from the same source, which were used for other experiments, is shown in Fig. 2.

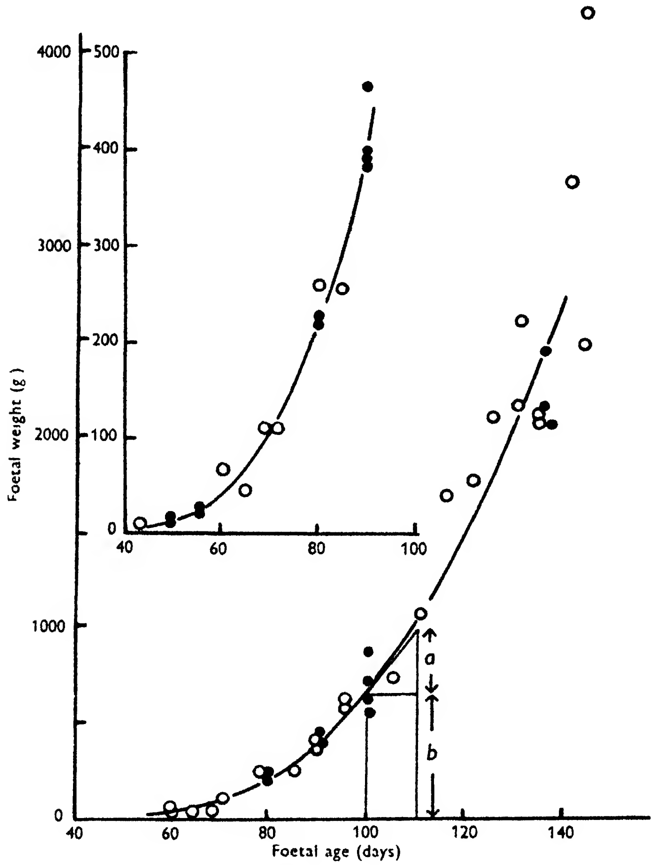


Fig. 2. Relationship of foetal weight to foetal age in the present series of sheep. Open circles represent singletons Solid circles represent twins The line used to compute the percentage increase in foetal weight per day has been drawn in. An example of the method of arriving at the daily percentage increase in foetal weight is shown. A tangent has been drawn to the curve at 100 days. The percentage increase in foetal weight in 10 days would be $100 \times a/b$, and the daily percentage increase would be $10 \times a/b$.

A line has been drawn through these points, which may be taken to represent the curve of weight increase for an average foetus. By drawing tangents to this curve at 10-day intervals, and making a geometrical construction of the type shown, it is possible to determine, at each age, the percentage increase per day in foetal weight of an average foetus. The percentage increase in foetal weight

per day at different ages has been calculated. These results are plotted in Fig. 3. The blood flow in ml./min./kg. of foetal weight is shown on the same figure, and the scales have been chosen so that the two sets of data correspond.

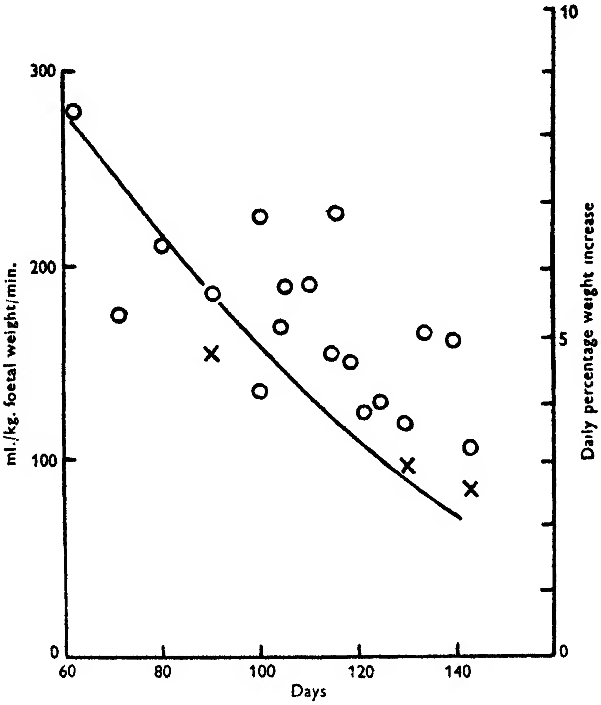


Fig. 3. Umbilical blood flow per kg. foetal weight at different foetal ages. Ordinate: umbilical blood flow. Abscissa: foetal age. Each circle represents the average of between four and ten observations on a single foetus. Crosses represent results obtained on foetuses in poor condition. Continuous line represents percentage increase in foetal weight per day, and the scale for this is at the right.

DISCUSSION

A number of indirect measurements of the rate of umbilical blood flow have been made by Barcroft. In 1939, Barcroft, Kennedy & Mason computed the blood flow in the umbilical cord from measurements of the foetal oxygen consumption and the arterio-venous oxygen difference in the umbilical vessels. These figures, with two exceptions, are lower than those now presented and are shown in Fig. 4. In 1934, Barcroft, Flexner & McClurkin measured the heart output in the goat. They then computed that the umbilical blood flow was between one-third and two-thirds of the cardiac output. This calculation was admittedly a rough computation based upon three major assumptions which in our view are untenable, namely, that (*a*) the whole of the blood leaving the heart for the foetal body is in one vessel, (*b*) that the venous blood returning to

the heart from the foetal body is fully reduced, and (c) that the oxygen content of the umbilical artery blood is the same as that leaving the heart for the foetal body. Nevertheless, if this computation of Barcroft, Flexner and McClurkin be applied to the cardiac output results of Barcroft & Torrens (1946), the vertical lines in Fig. 4 are obtained. It will be seen there is a remarkable agreement between these indirect results and the results of our own direct measurements. At the lower foetal ages their mean calculated flows would agree fairly closely with ours, and at the higher foetal ages their figures are rather lower than ours.

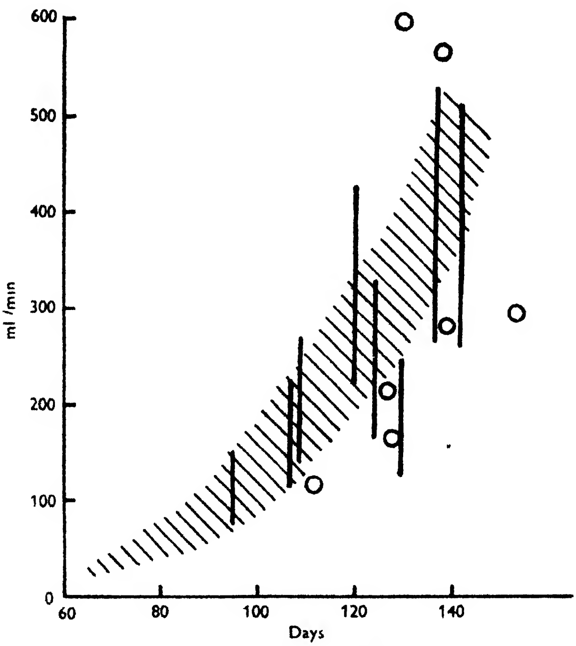


Fig. 4. Comparison of umbilical blood flow data with those of previous investigators. Ordinate: umbilical blood flow. Abscissa: foetal age. The upper end of each vertical represents two-thirds of the foetal cardiac output, and the lower end one-third, as measured by Barcroft & Torrens (1946). Circles—data of Barcroft, Kennedy & Mason (1939). Shaded area covers our own results, as shown in detail in Fig. 1.

It can be seen that there is a very fair agreement between the rate of blood flow and the percentage weight increase per day. This is particularly striking when it is remembered that no means exist for measuring the rate of increase for an individual foetus, and that large variations are thought to occur in the last part of pregnancy (Barcroft, 1946). It has also to be remembered that the proportion of the total foetal weight contributed by the various foetal organs is quite different at different foetal ages (Wallace, 1946, 1948; Carlyle, 1945).

These various organs will have quite different blood requirements for maintenance and growth. A precise relationship between the blood flow/kg./min. in the umbilical cord and the daily percentage increase in foetal weight is not, therefore, to be expected even if there is close agreement between the blood flow/kg./min. and the daily percentage increase in the weight of the individual organs of the foetus.

SUMMARY

1. The umbilical blood flow has been measured directly by the venous occlusion plethysmographic technique in sheep foetuses between the 60th and the 143rd day of intra-uterine life.

2. The umbilical blood flow/kg. foetal weight ranges from about 250 ml./min. at the earlier ages, to about 130 ml./min. at the later ages.

3. The relationship between the umbilical blood flow/kg. foetal weight/min. and the daily percentage increase in foetal weight at various foetal ages is presented, and it is concluded that there is some proportionality.

4. The results are compared with those obtained previously by indirect methods.

It is a pleasure to acknowledge the debt we owe to Prof. A. N. Worden of the Department of Animal Husbandry, University College, Aberystwyth, and his staff, particularly Mr Richard Phillips, Mr George England and Mr M. Fisher for the facilities provided by the University Farm at St Clears, by means of which it has been possible to obtain these two series of accurately dated pregnant ewes. Without the careful and painstaking work on the farm of Mr England, and later, Mr Fisher and their shepherd, these results would lose much in value.

It also gives us great pleasure to say how much we owe to Mr K. W. Grand, Assistant General Manager of the Great Western Railway and his staff of all grades, particularly Mr Gilbert Matthews, Station Superintendent at Paddington. The special transport facilities they provided have enabled us to maintain the closest liaison between the Universities of Wales and London, an essential requirement of this work.

We would thank our colleague, Sir Alexander Fleming, F.R.S., for the special adaptation of the Animal Houses of the Inoculation Department of this Medical School. We owe him much.

We wish further to acknowledge a generous grant to one of us (A. St G. H.) from the Halley Stewart Trust which has gone far to defray the expenses of the 1947 series of sheep.

Finally, we wish to thank the Medical Research Council for enabling us to pursue this work further on a larger and more accurate scale in 1948.

REFERENCES

Barcroft, J., Flexner, L. B. & McClurkin, T. (1934). *J. Physiol.* **82**, 498.

Barcroft, J., Kennedy, J. A. & Mason, M. F. (1939). *J. Physiol.* **95**, 269.

Barcroft, J. (1946). *Researches in Pre-Natal Life*, p. 33. Oxford: Blackwell.

Barcroft, J. & Torrens, D. S. (1946). *J. Physiol.* **105**, 22 *P*.

Carlyle, A. (1945). *J. Physiol.* **104**, 22 *P*.

Cooper, K. E. & Greenfield, A. D. M. (1948). *J. Physiol.* **108**, 167.

Greenfield, A. D. M. (1948). *J. Physiol.* **108**, 157.

Huggett, A. St G. (1927). *J. Physiol.* **62**, 373.

Wallace, L. R. (1946). Ph.D. Thesis, Cambridge.

Wallace, L. R. (1948). *J. agric. Sci.* **38**, 42.

J. Physiol. (1949) 108, 167–176 612.179.2

A METHOD FOR MEASURING THE BLOOD FLOW IN THE UMBILICAL VESSELS

By K. E. COOPER and A. D. M. GREENFIELD

From the Physiology Department, St Mary's Hospital Medical School, London

(*Received* 3 *March* 1948)

The blood flow in the umbilical cord has been measured directly by the use of a stromuhr (Cohnstein & Zuntz, 1884). This method is open to serious doubt because of the remarkable contraction of the walls of the vessels which may follow manipulation or irritation, and greatly alter the flow through them. Two indirect methods have been employed. In the first (Barcroft, Kennedy & Mason, 1939), the rate of foetal oxygen consumption was determined by observing the rate of decline of the oxygen content of the foetal arterial blood when the umbilical cord was clamped. The oxygen contents of the umbilical arterial and venous bloods were determined on another occasion, and the rate at which the blood would need to flow to supply the foetus with oxygen was calculated. In the second method (Barcroft, Flexner & McClurkin, 1934), the foetal cardiac output was measured by a cardiometer, and from an examination of the oxygen content of blood in various parts of the foetal circulation it was suggested that, allowing the validity of several assumptions, between one- and two-thirds of this quantity probably passed through the umbilical circulation.

It was thought possible that venous occlusion plethysmography, which has been applied to various parts of the peripheral circulation (Brodie & Russell, 1905; Hewlett & Van Zwaluwenberg, 1910; Grant & Lewis, 1925; Grant & Pearson, 1937), might be applied to the measurement of umbilical blood flow.

The criteria which must be fulfilled for this method to be admissible would be similar to those applying in other situations, namely:

(1) The technique used for obstructing the veins should be without local effect on the arteries.

(2) The cessation of venous return from the placenta should not disturb the foetal circulation sufficiently to alter (certainly in the initial period) the pressure in the umbilical artery. As the umbilical veins are thought to contribute between one- and two-thirds of the total venous return to the heart (Barcroft *et al.* 1934), this is a matter of prime importance.

(3) The foetal placental vessels must be sufficiently distensible to accommodate the blood accumulating in them during the early period of venous occlusion without developing a significant back pressure in the initial period.

It is easier for technical reasons to observe foetal rather than placental volume changes, and it is more satisfactory because there is only one circulation in the foetus, and there are two in the placenta.

METHODS

Apparatus. The type of plethysmograph used has been previously described (Greenfield, 1948*b*). The volume recorder used in the early experiments was of the float type with frontal point writing on smoked paper. This enabled the progress of an experiment to be judged while the technique was being worked out, but when confidence had been gained, an optically recording float recorder with more satisfactory properties was used. Both recorders were balanced to maintain all positions when open to the air.

Both types of recorder were dynamically calibrated while attached to the plethysmograph in the following manner. Mercury under a constant pressure head of about 100 cm. was allowed to flow away through a nozzle of adjustable size and, in doing so, drew saline from the plethysmograph through a tube in the position normally occupied by the umbilical cord (Fig. 1). At the commencement of an observation a large hole in the lid of the plethysmograph was left open to the air, and air was drawn into the plethysmograph through this hole rather than from the volume recorder. This hole was then closed as suddenly as possible, and thereafter the air was drawn into the plethysmograph from the volume recorder, causing it to be deflected at a rate corresponding to the steady flow of mercury from the system. The mercury was collected in a measuring cylinder for a carefully timed period, and the rate of flow so computed was related to the slope of the line made by the volume recorder.

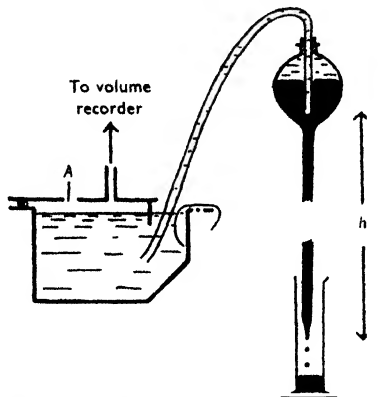


Fig. 1. Method used for dynamic calibration of the plethysmograph and volume recorder. Mercury, under a head of pressure *h*, about 100 cm., ran into a measuring cylinder through an adjustable nozzle. When a steady flow was attained, the hole in the lid of the plethysmograph at *A* was suddenly closed with a vaselined plate of Perspex, and the withdrawal of fluid from the plethysmograph thereafter caused the volume recorder to deflect.

It was found that the smaller plethysmograph coupled to the smoked paper volume recorder gave results over the range 0–300 ml./min. which were consistent to within $\pm 5\%$, and over this range there was a linear relationship between the rate of flow and the amount of deflexion in a given time. The large plethysmograph with photographic volume recorder was consistent within $\pm 3\%$ and linear in its deflexion over the range 0–600 ml./min.

Although these volume recorders were used for most of the experiments described in this paper, they have since been superseded by a soap-bubble volume recorder with greatly increased precision. (Greenfield, 1948*a*.)

Experimental. The observations were made on Welsh ewes of 30–45 kg. weight between the 60th and the 143rd day of pregnancy. The technique of preparation and Caesarean section was that of Huggett (1927), except that a spinal anaesthetic was used instead of urethane. The foetus after delivery was kept immersed in saline at 37° C. throughout the experiment.

The foetus was rotated if necessary to untwist the umbilical cord, and then placed in the plethysmograph (which was fixed between the hindlegs of the ewe) with the cord passing over the

rounded Perspex edge. Great care was taken to avoid pulling on the cord or touching it unnecessarily. The uterus was held towards the plethysmograph when necessary to keep the cord quite slack. The foetus was fixed loosely in position with lint packs and flexible metal bars. In some experiments barbs attached to electrocardiograph leads were inserted under the skin in the mid-line at the front and back of the chest.

The plethysmograph was adjusted so that the brim was about 1 cm. above the level of saline in the bath, and the saline in the plethysmograph was brought to the level of that outside. The lid was placed in position, care being taken not to nip the cord, and the plethysmograph and recording gear tested for leaks by manually depressing the float recorder for a few seconds and seeing that it returned on release to its original position.

The umbilical cord was passed over a finger stall on the brim of the plethysmograph, and this could be inflated very rapidly to any desired pressure by connecting it to a large air reservoir. The bag compressed the cord against a Perspex window, through which the vessels could be viewed. In the present experiments the cord was so compressed for periods of about 5 sec.

RESULTS

The first series of experiments established a technique for totally obstructing the umbilical veins without affecting the umbilical arteries.

In a small proportion of sheep umbilical cords the vessels have a sufficiently straight and parallel course to make them clearly and individually visible as they pass over the edge of the plethysmograph. In some cases the vessels can be arranged so that the two veins lie at the sides of the cord, and the arteries at the centre. When this is so, in the case of large cords, the veins can be digitally compressed, without applying pressure to the arteries. From this point of view, digital compression is perhaps the ideal method of obstruction, but it cannot be used on more than a small proportion of cords for anatomical reasons, and it often fails because the veins slip from under the finger. It was therefore clear that, for general use, pressure on the cord by an inflatable bag would be more convenient if it could be shown to act similarly.

The effects of various pressures in the bag used to compress the umbilical vessels. The following experiment was performed on ewe no. 151 with a 134-day foetus (one of twins) weighing 2120 g., which had a cord with exceptionally straight and parallel vessels. At intervals of about 30 sec. starting 6 min. after removal of the foetus from the uterus, twenty-six observations were made on the effects of using pressures between 20 and 90 mm. Hg in the bag. The scale of pressures was progressively ascended and descended twice. In every case the pressure was applied for about 5 sec. Direct observation of the arteries showed that they were considerably narrowed at 60 mm. Hg pressure, and emptied at 80–90 mm. Hg pressure. It was impossible to be certain whether or not they were at all narrowed at 40–50 mm. Hg pressure.

In each of the above cases the changes in foetal volume were recorded, and from these records, by means of the dynamic calibration already described, the initial rate of change of foetal volume was determined. The results are shown in Fig. 2. It will be seen that there is a maximum initial rate of change of foetal volume at 40–50 mm. Hg. At pressures greater than 40 mm. Hg, since

the veins were known by direct observation to be obstructed completely, the initial rate of change of foetal volume was clearly the same as the initial rate of blood flow in the umbilical arteries. The results show, therefore, that pressures greater than 50 mm. Hg caused a decrease in the flow through the umbilical arteries. The fact that the flows were substantially the same at 50 as at 40 mm. Hg suggests that the restricting influence of the bag pressure on the umbilical arterial flow did not operate below 50 mm. Hg, but further evidence on this point was provided by a comparison with digital compression.

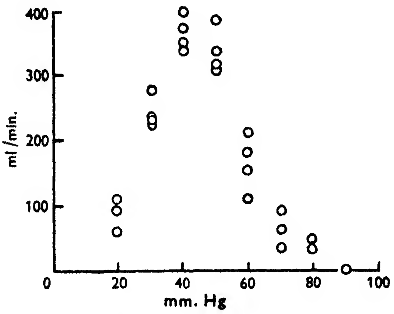


Fig. 2. Ewe no. 151.
134-day foetus.

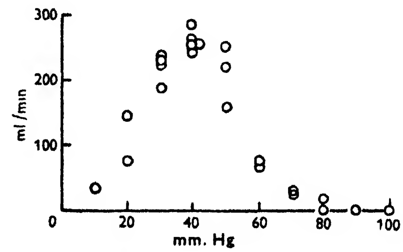


Fig. 3. Ewe no. 162.
130-day foetus.

Figs. 2, 3. Ordinate: initial rate of change of foetal volume in ml./min. following compression of umbilical cord. Abscissa: pressure in mm. Hg in the bag compressing the cord. Each point represents a single observation.

It may be noted in passing that the deduction may be made from Fig. 2 that the systolic blood pressure in the umbilical artery was between 80 and 90 mm. Hg, and the diastolic blood pressure was about 50 mm. Hg.

The results of a similar experiment on ewe no. 162 having a 130-day foetus of 2179 g. are shown in Fig. 3.

A less extensive experiment of the same type was performed on ewe no. 202 with an 80-day foetus weighing 255 g. It is presented because the foetus was less than one-eighth the weight of that just described. The results, which are given in Table 1, are similar. Fourteen observations were completed within 9 min. of removal of the foetus from the uterus, and pressures from 25 to 70 mm. Hg were employed. All were very satisfactory technically. The veins were completely occluded for the first 2 sec. or thereabouts when a 25 mm. Hg pressure was applied, and throughout the 5 sec. period with greater pressures.

The initial rate of change of foetal volume is considerably less at 40 than at 30 mm. Hg, between which pressures the diastolic umbilical arterial pressure presumably lies. The arteries were seen to be emptied of blood at 70 mm. Hg pressure; and the systolic pressure in them must lie between this figure and

65 mm. Hg. Over the range 25–35 mm. Hg there appears to be no interference with the flow through the arteries.

TABLE 1. Initial rate of change of foetal volume when the umbilical cord of ewe no. 202 was compressed by a bag at pressures of 25–70 mm. Hg, the umbilical veins being seen to be completely emptied over the bag in each case

Time from removal of foetus from uterus (min.)	Pressure employed in bag (mm. Hg)	Initial rate of change of foetal volume (ml./min.)
2	25	56
2½	35	49
3	25	49
4½	30	58
5	30	59
5½	35	56
6	40	37
6½	45	30
7	50	15
7½	48	32
7¾	55	15
8	60	26
8½	65	13
9	70	0

Comparison of the initial rate of blood flow in the umbilical arteries when the umbilical veins are obstructed by digital compression, and when the whole umbilical cord is compressed by a bag at a pressure sufficient to obstruct the veins. The same animal, no. 151, was used as in the first experiment, but this series of observations commenced 31 min. after removal of the foetus from the uterus. The results are shown in Table 2. It will be noted that the initial rates of umbilical

TABLE 2. Comparison of the initial rate of decrease of foetal volume when a bag inflated to 50 mm. Hg was used to compress the cord, and when the veins only were compressed digitally, ewe no. 151

Time from removal of foetus from uterus (min.)	Initial rate of change of foetal volume (ml./min.)	
	Bag compression	Digital compression
30·4	240	
31·6		315
34·0	235	
34·6		265
36·2	255	
36·7		330
37·3	300	
37·8		300
38·5	300	
38·9		340
39·6	340	
40·9		350
41·4	285	
42·2		340
42·7	285	
	Average 270	Average 320

arterial flow are somewhat less than in the previous experiment, using in each case a bag at 50 mm. Hg for venous obstruction. This is typical of the steady decline often seen in foetuses during a prolonged period of observation.

At intervals of about a minute the umbilical cord was compressed by a bag at 50 mm. Hg alternately with digital compression of the umbilical veins. Only if the operator was satisfied at the time that he had compressed the veins successfully, without pressure on the arteries, was account taken of the results of digital compression, but all results obtained by bag compression are presented. It will be seen that the averages of the flows observed following digital compression are about 15% higher than those using the bag at 50 mm. Hg.

A second experiment of the same type was performed on ewe no. 166 at 135 days, having a foetus (one of twins) of 2440 g. weight. This experiment was defective in that there is doubt about the absolute values of all the flows measured, but the relative proportions of these are probably reliable. In this case the average initial rate of flow in the umbilical arteries with 50 mm. Hg pressure in the bag was 3% greater than, and with 40 mm. Hg pressure in the bag was the same as, that observed when the veins were digitally compressed. In each case four observations have been averaged.

In a third experiment on ewe no. 136, at 130 days, having a foetus of 2600 g. weight, the following flows were observed when a bag at 35 mm. Hg (a sufficient pressure in this case) was used to obstruct the umbilical veins—285, 301, 278, 332, 308 ml./min. During this series, digital compression of the veins gave flows of 365 and 246 ml./min. In this case, although the averages are 301 and 305 ml./min. by the two methods, the scatter of the individual results detracts from their value.

A number of other isolated observations, supporting those quoted, lead to the conclusion that a bag inflated to a pressure just adequate to obstruct completely the umbilical veins has little if any effect in mechanically obstructing the umbilical arteries.

The second series of experiments and observations were made to discover whether the rate of blood flow in the umbilical arteries is immediately altered when the umbilical veins are obstructed. If it is not, the initial rate of flow following venous obstruction may be used as a valid measure of the normal rate of flow with the circulation undisturbed. The evidence on this point is indirect.

(*a*) Following venous obstruction the foetal volume decreases in a linear manner for 2 sec. or more (Fig. 4). This indicates that the umbilical arterial flow proceeds at a steady rate for the first 2 sec. It is unlikely that any influence producing a change at the instant of application of obstruction would produce no further progressive change during these 2 sec. The straightness of this part of the foetal volume curve further shows that the placental vessels accommodate the blood during this period without exerting notable back pressure.

(*b*) An electrocardiograph ink-writer trace enabled changes in foetal heart rate following obstruction of the umbilical veins to be followed very exactly. In later experiments the soap-bubble volume recorder (Greenfield, 1948*a*) clearly showed the individual pulse waves. It was found that the heart slowed, but that the slowing usually did not commence for 1–2 sec. after the umbilical veins had been obstructed. Four typical records obtained from the ewe

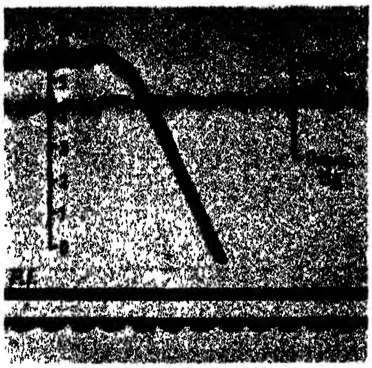


Fig. 4 *a*.

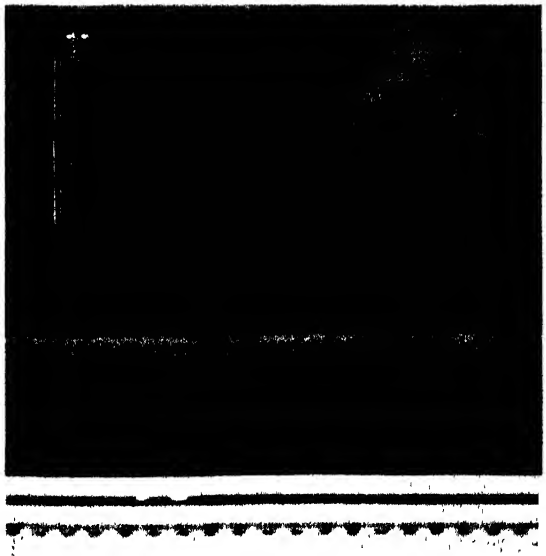


Fig. 4 *b*.

Fig. 4. Foetal volume curves showing the change on obstruction of the umbilical veins. In each case the undulations of the time trace are at the rate of 200/min., and the divisions on the vertical scale each represent 1 ml. (*a*) Curve obtained with photographic float recorder from ewe no. 136, 130-day foetus. This includes a simultaneous umbilical arterial pressure record taken from the umbilical artery between the foetus and the pressure bag, and the scale for this, in mm. Hg, is shown. (*b*) Curve obtained with soap-bubble volume recorder from ewe no. 218, 90-day foetus, showing pulse waves.

no. 151 at 134 days, are shown in Fig. 5. The heart rate, expressed as beats per minute, has been calculated for each beat (measured between successive *R* waves), and this has been plotted over a trace showing the foetal volume change when a 50 mm. Hg pressure in the bag is used to obstruct the umbilical veins. It can be seen that the slope of the foetal volume curve, and hence the rate of flow in the umbilical arteries, can be measured before there is any appreciable disturbance of foetal heart rate. Similar results were obtained on two other ewes. A close inspection of Fig. 4*b* reveals no detectable slowing of the pulse waves during the period of observation.

(c) The umbilical arterial pressure was measured in three ewes between the foetus and the occluding bag. To do this the cord was first treated with formalin, as described by Barcroft (1946), to prevent contraction of the vessels when a needle was inserted. A needle was then inserted, 1 ml. of heparin ('Liquaemin' 1000 units/ml.) injected, and the needle then placed in communication with a lead tube connected to an optically recording metal membrane manometer, the whole system being filled with saline to the exclusion of all air bubbles.

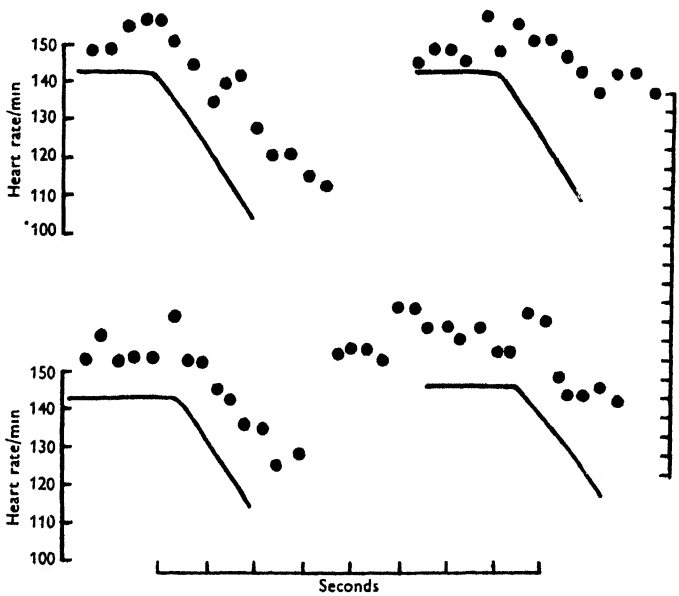


Fig. 5. Heart rate of each individual beat (dots) and foetal volume (continuous line) on obstruction of the umbilical veins. Ordinate: Heart rate, in beats/min. Scale without figures represents 1 ml. of volume per division. Abscissa: Seconds.

Umbilical arterial blood flows were measured with 50 mm. Hg pressure in the occluding bag before the application of formalin, after the application, and after the insertion of the needle, and it was established that these procedures had not disturbed the flow.

It was now possible to record simultaneously on the same photographic paper the foetal volume changes, and umbilical arterial pressure changes, produced by inflating the bag to 50 mm. Hg, thereby occluding the umbilical veins. Such a record appears in Fig. 4a. It can be seen that a slight rise of pressure occurs, but that the slope of the foetal volume curve can be measured before this takes place.

These experiments show that there is no sudden change in foetal heart rate or arterial blood pressure at the moment of umbilical venous occlusion, and it is therefore unlikely that there is, at this moment, any instantaneous change in

umbilical arterial blood flow, and that the umbilical arterial blood flow immediately following such occlusion is almost certainly the same as the blood flow in the undisturbed cord.

Temporary compression of the umbilical cord does not produce any effects, either locally in the cord, or generally in the foetal-placental circulation, which, while not invalidating a first observation, might cast doubt on subsequent observations. The evidence on this point is as follows:

(a) Foetal heart rate and arterial blood pressure return to resting level well within ½ min. of the release of the umbilical vein.

(b) Successive observations of umbilical flow commonly show a scatter of ±10% around the mean, but the first observation shows no constant tendency to be greater or less than those that follow. In some foetuses there is a slow decline in the general level of umbilical blood flow—in the case of no. 151 this amounted to a fall to about 75% of the initial value at the end of 1 hr. In other cases no such decline is seen, and in the case of no. 126 there was no decline at all over a period of 30 min. during which thirty observations were made. It is clear, therefore, that the fact of making observations is not responsible for the decline in general foetal condition, and no disturbance of more than half a minute's duration is known to follow the few seconds of umbilical venous occlusion.

DISCUSSION

It appears that the technique of venous occlusion plethysmography can be successfully applied to the measurement of the umbilical blood flow, provided that an occluding pressure is employed just adequate to obstruct the umbilical veins for 3–5 sec. and suitable plethysmograph and recording gear are employed.

The method has the advantage that it can be carried out on the intact umbilical cord, and does not produce more than a momentary disturbance in the foetal or placental circulations. Repeated observations are possible—in one case over 100 have been made on one foetus.

The disadvantages of the method are that the foetus must be enclosed in the plethysmograph while each observation is made, although it is reasonably accessible between observations, and it is necessary to open the uterus to make the measurement. It is not known to what extent this factor may disturb the umbilical circulation. The only means of obtaining even indirect evidence on this point would appear to be a comparison of the foetal heart rate in utero and in the plethysmograph. This has not yet been done.

A very high degree of accuracy is attainable when the plethysmograph is used with a soap-bubble volume recorder.

It is likely that results obtained by this method, if they err at all, err on the side of being too low rather than too high.

SUMMARY

1. A method has been developed which enables the rate of blood flow in the umbilical arteries to be measured repeatedly at short intervals, after removal of the foetus from the uterus.

2. Observations may be commenced within 1 min. of removal from the uterus.

3. The relationship between the blood flow in these conditions and that in utero is not known.

We are very grateful to Prof. A. St G. Huggett for arousing our interest in foetal physiology, and for giving us the opportunity to establish this method on his series of pregnant ewes. We wish to thank Dr D. McK. Kerslake for assistance in the early experiments.

REFERENCES

Barcroft, J., Flexner, L. B. & McClurkin, T. (1934). *J. Physiol.* **82**, 498.
Barcroft, J., Kennedy, J. A. & Mason, M. F. (1939). *J. Physiol.* **95**, 269.
Barcroft, J. (1946). *Researches in Pre-Natal Life*, p. 192. Oxford: Blackwell.
Brodie, T. G. & Russell, A. E. (1905). *J. Physiol.* **32**, xlvii.
Cohnstein, J. & Zuntz, N. (1884). *Pflüg. Arch. ges. Physiol.* **34**, 173.
Grant, R. T. & Lewis, T. (1925). *Heart*, **1**, 87.
Grant, R. T. & Pearson, R. S. B. (1937). *Clin. Sci.* **3**, 119.
Greenfield, A. D. M. (1948*a*). *J. Physiol.* **107**, 17 *P*.
Greenfield, A. D. M. (1948*b*). *J. Physiol.* (in the Press).
Hewlett, A. W. & Van Zwaluwenberg, J. G. (1910). *Heart*, **1**, 87.
Huggett, A. St G. (1927). *J. Physiol.* **62**, 373.

J. Physiol. (1949) 108, 177–185 612.646

THE PASSAGE INTO THE EMBRYONIC YOLK-SAC CAVITY OF MATERNAL PLASMA PROTEINS IN RABBITS

By F. W. ROGERS BRAMBELL and W. A. HEMMINGS

From the Department of Zoology, University College of North Wales, Bangor

WITH AN ADDENDUM ON

Electrophoretic and Ultracentrifugal Examination of Rabbit Blastocyst Fluid

By E. F. McCARTHY and R. A. KEKWICK

From the Lister Institute of Preventive Medicine, London

(*Received* 12 *March* 1948)

In wild rabbits many litters are lost by the death of all the embryos between the 11th and 15th days of gestation. The presence of a fibrin clot in the yolk-sac cavity in many 7-, 8-, 9- and 10-day embryos might be connected with their subsequent death (Brambell & Mills, 1946, 1947). The presence of fibrinogen in the yolk-sac fluid of tame rabbit embryos at corresponding stages was demonstrated experimentally in a concentration approximately one-third that of the maternal plasma. The yolk-sac fluid from which the fibrin had been removed contained slightly more than one-half the amount of nitrogen in the maternal serum, indicating the probable presence of other unidentified proteins. The presence of substantial quantities of fibrinogen in the yolk-sac at such early stages, when the embryonic heart is only beginning to beat, suggested that it must be of maternal origin. The experiments described herein were performed to test this suggestion.

Some means of marking the plasma proteins in the maternal circulation was required so that they could be identified subsequently in the yolk-sac fluid of the embryo if they passed into it. Prof. John Beattie, to whom we are greatly indebted, suggested that the dye Evan's blue (T. 1824) might prove suitable. This dye is not toxic and can be injected intravenously in considerable quantities. It can be identified spectroscopically in body fluids in very low concentrations. Efskind (1940) states that this dye forms a relatively stable compound with the albumin fraction of rabbit plasma. Rawson (quoted by Abramson, Moyer & Gorin, 1942) observed the preferential binding of the dye, T. 1824, with the albumin fraction of human and dog serum and plasma. He showed by electro-

phoresis and ultracentrifuge studies that up to 8 mol. dye are bound by 1 mol. albumin. At higher concentrations some of the dye migrates with the α- and β-globulins in addition to the albumin.

Preliminary experiments performed by one of the authors (F. W. R. B.) in conjunction with Dr I. H. Mills showed that this dye passes rapidly into the yolk-sac fluid of 8-day rabbit embryos when injected intravenously into the maternal circulation. A series of experiments with Evans blue was therefore undertaken in the hope that it would throw light on the problem of whether maternal plasma proteins pass through the embryonic omphalopleur into the yolk-sac cavity. The results led to the biophysical investigation of the protein content of the yolk-sac fluid and of the maternal plasma by Dr R. A. Kekwick and Dr E. F. McCarthy, whose co-operation rendered this part of the work possible.

The structure of the omphalopleur and its relation to the mucosa in the rabbit have been reinvestigated by Miss Patricia Allen in this Department and the following account is based on her, as yet unpublished, report. The blastocyst of the rabbit at the beginning of the 7th day post-coitum is a spherical vesicle approximately 3·5 mm. in diameter. It is surrounded by the stretched and attenuated zona pellucida and lies free in the uterine lumen, but it is orientated so that the embryonic shield is at the mesometrial pole. The entoderm has extended beneath the trophoblast around rather less than the embryonic hemisphere. Implantation occurs early on the 7th day, after the blastocyst has escaped from the ruptured zona pellucida, the trophoblast becoming attached to the uterine mucosa at a number of isolated points over the antimesometrial hemisphere. The blastocyst expands rapidly during the two succeeding days and attains a volume of 0·75 c.c. by the end of the 9th day, the increase of nearly 70 times in volume being due mainly to increase of the fluid content of the yolk-sac. During this period the trophoblast of the anti-mesometrial hemisphere has been invading and eroding the uterine mucosa, each attachment point acting as a centre from which the invasion radiates. The entoderm extends rapidly within the trophoblast and encloses the yolk-sac cavity. Meanwhile the amniotic folds have been forming around and closing over the embryonic shield at the mesometrial pole, and the chorionic trophoblast covering the outer walls of these folds becomes attached to, and invades, the mucosa of the placental ridges even before the closure of the amnion is completed on the 9th day, thus establishing the rudiment of the future allanto-chorionic placenta. The increasing thickness of the placental rudiment results in a flattening of the mesometrial side of the blastocyst, which becomes more nearly hemispherical than spherical by the end of the 9th day. The sinus terminalis, marking the limit of the extension of the mesoderm, surrounds the flattened placental area; elsewhere the bilaminar omphalopleur, forming the outer wall of the yolk-sac, is adjacent to the mucosa. The embryonic heart has been observed to be beating by 9½ days post-coitum. By the 15th day the allanto-chorionic placenta is fully established and the bilaminar omphalopleur has disappeared, opening the yolk-sac cavity to the uterine lumen and freely exposing the entoderm of the splanchnic wall, which has been inverted by the expansion of the amnion and exococle (Mossman, 1926).

METHODS

The animals used in these experiments were mainly, but not exclusively, the Agricultural Research Council's Compton strain of Dutch rabbits. The majority were well-grown young females pregnant for the first time. Mating was watched and the buck was allowed to fall twice, the doe being then immediately removed from his cage.

Evans blue (supplied by British Drug Houses Ltd.) in saline solution containing 10 mg./ml. was employed. A standard dose of 3 ml. was injected into the ear vein and the animal was killed 1–2 hr. later. It was found that uterine contractions after the animal was killed, intensified by manipulation of the uterus, were liable to cause shrinkage of the blastocysts through loss of fluid or even rupture. Since precision in the measurement of the permeability of the omphalopleur depends upon the accuracy of the measurements of the volume of yolk-sac fluid, as well as upon the estimation of the concentration of the dye, this presented a serious technical difficulty. Indeed, the experimental error arising from loss of yolk-sac fluid through uterine contraction is of a greater order of magnitude than that arising from any other cause. We have devoted much attention to this problem and tried a variety of methods of killing but we have not yet achieved perfection. An ideal method would need to be sufficiently rapid, relative to the period over which the measurement is made, for any effect it might have on the permeability of the tissues to be of negligible duration, and also the uterine musculature must be rendered relaxed and inexcitable. Killing by a blow on the back of the head was used at first, but discarded because of uterine contractions. Ether anaesthesia was discarded because of its probable effect on permeability over a relatively long period owing to its slow action, as was also urethane in a lethal dose administered subcutaneously, although the latter leaves the uterus relatively inactive. A lethal dose of veterinary Nembutal (65 mg. in 2 ml. aq. dist.) administered intravenously, kindly suggested to us by Prof. J. H. Gaddum, is almost instantaneous but does not leave the uterus sufficiently inactive. The most satisfactory method employed so far is that of Meltzer & Auer (1908) which consists of 0·5 ml. M/8 solution of calcium chloride injected intravenously (i.e. half the dose required to prevent respiratory paralysis) plus 10 ml. per kg. body weight M/1 solution of magnesium chloride injected subcutaneously. This kills in about 10–20 min. and leaves the uterus fairly relaxed and inexcitable. It is neither as quick, nor is its effect on the uterus as complete, as we would like, but it is a considerable improvement on the other methods mentioned.

The abdomen was opened immediately after death, and the size of the blastocysts estimated visually by comparison with a series of wax models of uterine swellings of 1 mm. in diameter intervals. The uterus was then ligatured on each side of each swelling, dissected out whole, and the swellings again compared with the models to determine if there had been perceptible shrinkage. It was then cut into segments, each including one swelling and the ligatures on each side of it. Each swelling was immediately dropped into a tube of iso-pentane cooled in a thermos flask containing solid carbon dioxide in 70% alcohol and rapidly frozen solid. Subsequently, the frozen swellings were taken out, the anti-mesometrial uterine wall slightly thawed in the fingers, slit with a knife, and the blastocyst shelled out as a little globule of ice, free from uterine tissue. This was weighed in a tube while still frozen hard, and then allowed to thaw after the requisite amount of a 1·6% solution of sodium oxalate had been added. The oxalated fluid was then spun in a centrifuge, to throw down any fragments of the embryonic membranes remaining, and the supernatant fluid pipetted off. This method of collection of the fluid was found to be far better than aspiration with a syringe, as it reduced to a minimum the risk of loss of fluid or of appreciable contamination with tissue exudates in the uterine lumen.

The concentration of dye in the yolk-sac fluid was estimated by comparison with a standard series of aqueous solutions of the dye on a Hilger Specker Photoelectric Absorptiometer, micro-model, using an Ilford Yellow 606 filter. Samples showing any yellow owing to contamination with traces of blood were discarded, so that it was unnecessary to use a less sensitive filter with a transmission band beyond the absorption range of haemoglobin to exclude the effects of haemolysis, as is essential when estimating plasma samples. Corrections for turbidity and for the slight shift in the absorption band of the dye in the presence of proteins, are omitted for simplicity, since the magnitude of the errors arising therefrom are negligible for the purposes of the present paper.

RESULTS

The maternal blood. The amount of dye injected into the maternal circulation was 30 mg., irrespective of body size. The total volume of blood in a rabbit is related to its size (Dreyer & Ray, 1910) and may be roughly estimated at 100–120 ml. for the Dutch rabbits employed. Hence the initial concentration of dye in the blood after injection will have been 250–300 mg./l., but the concentration falls subsequently by approximately 23 % in the first hour and by 27 % in the first 2 hr. Therefore the proportion of dye to albumin cannot at any time have risen above a molecular ratio of 1:1.

A sample of blood was collected from the heart of a large rabbit (13711) 8 days 20 hr. pregnant, 2 hr. after injection of the dye, under ether anaesthesia. The blood was oxalated, cooled, centrifuged and the plasma examined by Drs Kekwick and McCarthy. They found that in the ultracentrifuge the dye sedimented quantitatively with the proteins, leaving a colourless supernatant fluid. Since minute quantities of the dye can be detected visually it is clear that it was all attached to the proteins. It appeared, from the diagram, that the dye was combined with the albumin, but no evidence could be obtained on whether or not it was combined also with the globulin. The plasma accordingly was examined electrophoretically in the U-tube. This confirmed the evidence from the ultracentrifuge that the bulk of the dye was combined with the albumin, though it was possible that a small amount may have been combined with the α-globulin. No dye was combined with the fibrinogen nor with the β- and γ-globulins.

The passage of dye through the omphalopleur. The concentration of dye in the yolk-sac fluid of the blastocysts of ten rabbits 7 days 20 hr. to 8 days 23 hr. post-coitum are given in Table 1. The fall in concentration with increasing age

TABLE 1. Concentration of dye (mg./l.) in yolk-sac fluid.

Ref. no. of rabbit	Age (days and hr.) at death (post-coitum)	Embryo no. 1	2	3	4	5	6	7	8	Mean
13744	7.20	33·0	29·0	20·0	19·5	17·0	—	—	—	23·7
13818	7.22	15·5	14·5	14·0	12·5	12·5	12·0	11·5	—	13·2
23419	8.00	13·5	12·5	12·0	11·5	10·0	—	—	-	11·9
083	8.03	30·0	19·0	19·0	17·0	16·0	11·5	—	—	18·8
13748	8.06	13·5	13·0	12·5	11·0	8·5	6·5	—	—	10·8
13743	8.09	9·5	9·0	8·5	8·5	8·0	7·0	5·5	5·5	7·7
13705	8.14	6·5	5·0	4·5	—	—	—	—	—	5·3
13706	8.16	6·5	5·5	5·0	5·0	4·5	4·5	—	—	5·2
13711	8.20	3·5	3·0	—	—	—	—	—	—	3·3
13749	8.23	Nil	Nil	Nil	Nil	—	—	—	—	0·0

is to be expected, since the fluid passing in after injection will be diluted by the amount of fluid contained in the yolk-sac at the time of injection. Since these animals were all killed by a blow on the head, except for one (13711) which was

killed with ether, it is thought probable that fluid was lost before collection by uterine contraction; consequently it is not permissible to use them for estimates of the rate of passage of the dye through the omphalopleur, for which purpose the quantity of yolk-sac fluid, as well as the concentration in it, must be determined. This consideration does not invalidate the estimates of concentration, which show clearly that substantial quantities of the dye pass from the maternal circulation into the yolk-sac cavity at the time when the blastocyst is expanding most rapidly.

It was repeatedly observed that the embryonic membranes through which the dye must have passed were not appreciably stained, even when the dye had attained a high concentration in the contained fluid. Owing to the affinity of the dye for proteins, when free in solution, this suggested that it has passed through the membranes in a state of combination without becoming detached.

The yolk-sac fluid. Electrophoretic examination of the yolk-sac fluid requiring 10 ml. of a 2% protein solution involves the use of a combined sample of fluid collected from a number of blastocysts from several pregnant uteri. Since the concentration of protein in the undiluted yolk-sac fluid is known to be of the order of 50% of that in plasma, or approximately 4%, a sample of not less than 5 ml. yolk-sac fluid is required. This should be obtained from ten blastocysts 8 days post-coitum or two pregnant uteri; allowing a margin for small or abnormal blastocysts and some wastage in collection, three pregnant uteri are generally required. Three experiments were performed.

Experiment I. Yolk-sac fluid containing Evans blue was collected from two pregnant animals (13761 and 13762) 7 days 20 hr. and 8 days 22 hr. post-coitum respectively. Both animals were killed with urethane, which there is reason to believe had increased the permeability of the membranes, permitting more blue to pass into the yolk-sac fluid than would have been the case otherwise. The fluid was oxalated and diluted for estimation of the blue, the mean concentration being found to be 15·5 and 6·7 mg./l. respectively.

Experiment II. 5·2 ml. of yok-sac fluid was collected from four rabbits (13716, 13719, 13722 and 13766) 8½ days post-coitum and oxalated. These animals had not received dye and were killed by Nembutal.

Experiment III. 10·1 ml. of yolk-sac fluid was collected from three rabbits (13776, 13777 and 13780) 9 days 4 hr. post-coitum and oxalated. These animals had not received dye and were killed by magnesium chloride by the method described.

All three samples of yolk-sac fluid were found to be electrophoretically almost identical with rabbit plasma, showing the same number and general quantitative distribution of components. The sample containing the dye was a little too dilute to give the best results, but at least the greater part of the dye, if not the whole, migrated with the albumin, as in plasma. Ultracentrifuge examination of one sample of the undialysed yolk-sac fluid indicated that no low molecular products of the breakdown of proteins were present.

DISCUSSION

The results of the examination of the yolk-sac fluid show that it contains all the principal protein components of the maternal plasma, fibrinogen, albumin, α-, β- and γ-globulins, and that they are present in closely similar ratios to those of the plasma. The concentration is in the vicinity of 50% of that of plasma. It would be exceedingly difficult to interpret this similarity otherwise than by supposing the proteins of the yolk-sac fluid to be derived directly from the maternal circulation without being broken down and resynthesized in passage. Moreover, it is necessary to assume further, in view of the constancy in proportion of the various categories of proteins, that no appreciable differential selection occurs.

It has been shown previously (Brambell & Mills, 1946, 1947) that fibrinogen occurs in the yolk-sac fluid and that it clots when human thrombin is added to the oxalated fluid. A clot is also rapidly formed in yolk-sac fluid in vitro if it is not oxalated. Its behaviour is, in these respects, identical with the fibrinogen of the maternal blood. Clots of fibrin may be formed in certain circumstances in vivo in the yolk-sac cavity, especially in wild rabbit embryos, and can be detected histologically. It has not been found possible to collect fluid from the amnion or exocoele of embryos at such early stages of development, when these cavities are but recently formed, and to test directly for the presence of fibrinogen in them; but the histological evidence that fibrin clots have not been observed in them, even when conspicuous in the yolk-sac, appears to show conclusively that fibrinogen is not present in appreciable quantities. Since the mesoderm is the only constant component of the membranes bounding these cavities which is absent from the bilaminar omphalopleur, it may provide a barrier to the passage of fibrinogen.

The apparent absence of fibrinogen from the exocoele renders it improbable that maternal fibrinogen could reach the yolk-sac cavity through the yolk-sac splanchnopleur. It may be assumed, therefore, that it does so through the bilaminar omphalopleur which forms the whole of the remainder of the yolk-sac wall after the completion of the entoderm on the 7th day. This is in any case the more obvious route, forming as it does the greater part of the yolk-sac wall and virtually the whole of that part of it which is in direct contact with the uterine tissues. The omphaloidean trophoblast is in continuity at many points over the anti-mesometrial hemisphere with the vascular subepithelial tissues of the uterine mucosa.

The behaviour of the dye, Evans blue, is interesting. The whole of this dye in the maternal blood, even in the massive concentrations employed, is attached to the serum proteins, and all, or almost all, of it to the albumin. It is possible that a small proportion of the dye may be attached to the α-globulin. These results, obtained by means of the ultracentrifuge or by electrophoresis, confirm

and extend those of Efskind (1940) and of Rawson (quoted by Abramson *et al.* 1942). The dye in the yolk-sac fluid, which it has reached from the maternal circulation, is similarly attached. The fact that the embryonic membranes through which the dye has passed to reach the yolk-sac cavity remained inappreciably stained by it, suggests that it retained its attachment to the maternal serum albumin during its passage. It must be concluded, therefore, that the maternal plasma proteins can, and do, pass in considerable quantities into the yolk-sac cavity through the bilaminar omphalopleur of the rabbit embryo on the 7th and 8th days post-coitum, that they retain their identity whilst doing so, and that there is no convincing evidence of differential permeability of the membrane to the various kinds of proteins involved.

The blastocyst is expanding rapidly, mainly owing to the accumulation of yolk-sac fluid, during the 7th and 8th days. Although the blastocyst is a very thin-walled vesicle, it retains its globular form throughout this period, only becoming flattened on the mesometrial side where the greater thickness of the maternal tissues might be expected to present a greater resistance to its expansion. This expansion causes the anti-mesometrial side of the uterus to be blown out like a bubble. The problem therefore arises as to how the vesicular blastocyst can expand at a time when protein molecules are passing relatively freely through its wall. Further researches are in progress to elucidate the mechanism involved.

The functional significance of the maternal plasma proteins to the embryo presents another problem. The yolk-sac is morphologically part of the embryonic gut, being lined with entoderm, and is phylogenetically an absorptive organ. It would appear probable therefore that the plasma proteins may be utilized for the nutrition of the embryo, being a principal component of the histiotrophe, but they do not appear to be broken down in the yolk-sac cavity, since the ultracentrifuge revealed no significant amounts of degradation products.

SUMMARY

1. A technique for collection of yolk-sac fluid from rabbit embryos for analysis is described.

2. When Evans blue is injected intravenously, all the dye, up to the limits of the massive doses employed, is attached to the serum proteins, mainly or entirely to the albumin, although possibly some may be attached to the α-globulin.

3. This dye passes rapidly from the maternal circulation into the yolk-sac fluid where it is still attached to the serum proteins. The dye scarcely stains the embryonic membranes through which it passes, suggesting that it remains attached to the serum proteins during its passage into the yolk-sac.

4. The yolk-sac fluid contains the same number and general distribution of protein components as the maternal plasma.

5. It is concluded that the maternal plasma proteins pass in considerable quantities into the yolk-sac cavity through the bilaminar omphalopleur of rabbit embryos on the 7th and 8th days post-coitum, that they retain their identity whilst doing so, and that there is no convincing evidence of differential permeability of the membrane to the various kinds of proteins involved.

We have pleasure in acknowledging the advice and assistance we have received from our colleagues, Prof. E. D. Hughes and Dr T. Dostrovsky, in relation to absorptiometric measurements and in the provision of supplies of solid carbon dioxide, from Mr W. T. Rowlands on haematological technique, and from Miss M. Henderson, Miss Patricia Allen and Miss Helen Parry who are engaged on other aspects of these researches, in addition to those already referred to in the text. The work forms part of a larger scheme of research on prenatal mortality being conducted in this department and financed by a grant from the Agricultural Research Council, for which we wish to express our thanks.

REFERENCES

Abramson, H. A., Moyer, L. S. & Gorin, M. H. (1942). *The Electrophoresis of Proteins.* New York: Reinhold Publishing Corporation.

Brambell, F. W. Rogers & Mills, I. H. (1946). *Nature, Lond.*, **158**, 24.

Brambell, F. W. Rogers & Mills, I. H. (1947). *J. exp. Biol.* **23**, 332.

Dreyer, G. & Ray, W. (1910). *Philos. Trans.* B, **201**, 133.

Efskind, L. (1940). *Acta med. Scand.* **103**, 382.

Meltzer, S. J. & Auer, J. (1908). *Amer. J. Physiol.* **21**, 400.

Mossman, H. W. (1926). *Amer. J. Anat.* **37**, 433.

ADDENDUM

By E. F. McCARTHY and R. A. KEKWICK

Three samples of pooled blastocyst fluid were examined. One of these contained Evans blue, and a corresponding sample of maternal plasma also containing Evans blue was similarly studied.

The ultracentrifugal and electrophoretic examinations showed that blastocyst fluid and plasma are very similar in protein constitution. Blastocyst fluid showed two components in the ultracentrifuge sedimenting in the same manner as those of plasma, and electrophoretically it exhibited components corresponding in behaviour to the plasma components, albumin, α-, β- and γ-globulin and fibrinogen. Since the blastocyst fluid was obtained from more than one animal the possibility of a direct comparison with the maternal plasma was excluded. The electrophoretic analysis of the blastocyst fluid and rabbit plasma and serum are given in the table.

TABLE 1. Electrophoretic analyses

	Albumin	Globulins			Fibrinogen
Blastocyst fluid	64·0	4·0	12·5	12·8	6·6
Rabbit plasma (1)	63·3	11·5	13·0	4·3	7·9
Rabbit serum (2)	76·0	1·1	10·8	12·2	—

Amounts given are percentages of the total protein.

In the plasma containing Evans blue almost all the dye was attached to the albumin, with possibly a trace on the α-globulin, none remaining uncombined with protein. The dye in the blastocyst fluid behaved in precisely the same manner.

The ultracentrifugal examination of an undialysed sample of blastocyst fluid gave no evidence of the presence of significant amounts of low molecular material which might be associated with protein degradation.

METHODS

Unless otherwise stated samples were dialysed to equilibrium against phosphate buffer pH 8, $\mu = 0{\cdot}2$ at 1° C. The electrophoretic examination was made in the Tiselius apparatus at 1° C., protein refractive increment 0·00300 ($\lambda = 546$ mμ.) with optical observation by the Philpot diagonal Schlieren system. To dialysed solutions used in the Svedberg oil turbine ultracentrifuge, sodium chloride was added to a concentration of 0·15M.

REFERENCES

(1) Deutsch, H. F. and Goodloe, M. B. (1945). *J. biol. Chem.* **161**, 1.
(2) Seibert, F. & Nelson, J. W. (1942). *Proc Soc. exp. Biol., N.Y.*, **49**, 77.

J. Physiol. (1949) 108, 186–196 612.017.4:612.172.2

BRADYCARDIA OF CENTRAL ORIGIN PRODUCED BY INJECTIONS OF TETANUS TOXIN INTO THE VAGUS NERVE

By N. AMBACHE and O. C. J. LIPPOLD

From The Department of Physiology, University College, London

(*Received* 5 *April* 1948)

Although it is probable that functional derangements within the autonomic nervous system may occur in tetanus (Courtois-Suffit & Giroux, 1918), they are usually overshadowed, in the general clinical picture, by the severe disturbances in the somatic nerves and their centres. Harvey (1939) has put forward the thesis that tetanus toxin produces a pathological change in the process of cholinergic transmission at motor nerve-endings, with apparent overactivity. If we accept his point of view there seems to be no reason why the vagus nerve should not exhibit a similar process resulting in bradycardia. Clinically, however, there is no evidence for this; in fact the reverse appears to be the case according to Dean (1917), who reported tachycardia (without pyrexia) in general tetanus. Experimentally this is also usually the case, unless the toxin is injected directly into the vagus nerve. This particular experiment was first performed by Meyer & Ransom (1903) on two dogs both of which developed bradycardia. The same phenomenon is obtainable in rabbits, and we have analysed this effect, which appears to be due to a central action of the toxin on the vagal nucleus.

METHODS

The preparation and standardization of the CN 655 toxin, which was used for these experiments, has been described previously (Ambache, Morgan & Payling Wright, 1948). As before, the toxin was dissolved in sterile saline (0·9% NaCl), in the majority of experiments immediately before each injection.

The experiments were all performed on rabbits of mixed stock and weighing between 0·45 and 3·2 kg. After the initial heart rate was measured at rest, the animals were anaesthetized with 26 mg./kg. body weight, of 'veterinary nembutal' (pentobarbital sodium) intravenously. The toxin, in doses ranging from 20 μg. to 1 mg., was injected with aseptic precautions either into the vagus nerve itself or in close approximation to it, in one of the following ways: (*a*) The vagus nerve was exposed in the neck and carefully separated from the common carotid artery. The nerve was raised gently on a sterile loop of silk thread at the level of the cricoid cartilage. A very fine short-bevelled syringe needle (no. 27 S.W.G.) was then inserted through the perineurium and enough toxin solution

(0·05–0·1 c.c.) was injected in a peripheral direction to produce a small visible swelling within the nerve. The toxin was injected into one or other vagus nerve except in one animal in which it was injected into both vagi. In three rabbits which served as controls for (*a*) and (*b*) a mixture of toxin and antitoxin was injected centripetally into one vagus. The toxin for these controls was neutralized, just before the injections, with a 150-fold excess of antitoxin (Wellcome tetanus antitoxin globulins). (*b*) The vagus nerve was exposed on one side in the neck. It was firmly ligated and cut distal to the ligature; 2–3 in. of the peripheral end were avulsed. Toxin was then injected upwards into the ligated central end. An ascending flow of the toxin solution, which is yellowish, was clearly visible within the perineurium at the time of the injection and a small bubble of air from the dead-space of the syringe-needle was sometimes seen disappearing out of sight within the nerve. (*c*) The toxin was injected into the carotid sheath, away from the vagus and without touching the nerve. (*d*) The abdomen was opened by a midline incision and the stomach and oesophagus were delivered through the wound. After the vagus nerves were identified on either side of the oesophagus, the syringe needle was inserted through the anterior peritoneal covering of the oesophagus. The toxin (mixed with indian ink in the first few experiments) was injected subperitoneally (i.e. between the peritoneum and the outer muscular layer of the oesophagus) in an upward direction, in a volume of saline of 1–2 c.c. Contact with the vagi was carefully avoided throughout this procedure. The control animals received either (i) similar amounts of toxin boiled for 2 min. or (ii) saline, or (iii) 1 mg. of toxin neutralized immediately before the injection with a 150-fold excess of antitoxin. (*e*) The stomach was delivered as in (*d*), but the toxin (mixed with indian ink in some of the experiments) was injected subperitoneally at 3–4 loci along the lesser curvature and on the anterior wall of the stomach. The injection (volume 1–2 c.c.) raised a small swelling between the peritoneum and the outer muscular layer of the stomach. Again, contact with the branches of the anterior vagus was avoided.

After the animals recovered from the operation, they were examined at least once a day until their death. The apex beat was counted by auscultation through a stethoscope; several (from 5–10) consecutive 5 sec. counts were recorded. The mean of these was taken in each case as the heart rate per min.

The heart rate was recorded electrically in some of these animals, with the help of a direct writing 'Cardiotron' electrocardiograph for the loan of which we are indebted to Messrs Phillips and Co. As the input to this instrument is balanced symmetrically with respect to earth, interference from the a.c. mains, and the need to screen the animal are eliminated. A chest lead was found in trial experiments to be the most satisfactory. Records taken with other leads (I and II) showed considerable attenuation of the cardiac potentials. Before the operation the fur was removed, by shaving, from an area about 3–4 cm. in diameter on (*a*) the left anterior, (*b*) the right posterior chest wall and (*c*) on one thigh. At the time when records were taken an abrasive electrode jelly was rubbed into these areas and the three leads were either clipped on to the skin or strapped on tightly. The thigh lead was earthed and the two chest leads were connected to the input stage. E.c.g.'s were taken before, and at various intervals after, the injection of toxin.

RESULTS

Injections of toxin into the cervical vagus

The injection of toxin into an otherwise intact vagus nerve, or into the central end of a cut vagus, on either side, brings about, after a latent period of 22–43 hr., a considerable slowing of the heart (see Table 1). In the eight experiments listed in Table 1A, the mean maximal fall in heart rate was 176 beats/min. from an initial mean rate of 292/min. The onset of this bradycardia may be quite rapid; e.g. in Exp. 6 the heart rate dropped by 172 beats/min. in the 5 hr. between the two measurements taken at 17 and 22 hr.

TABLE 1. Bradycardia in rabbits after injections of tetanus toxin into the vagus nerve in the neck

The heart rates recorded in this and subsequent tables were measured initially before anaesthesia and injection, and at various intervals specified in parentheses, after injection.

Rabbit no.	Wt. (kg.)	Dose of toxin (mg.)	Site and direction of injection	Resting heart rate (beats/min.) Initial	After injection (hr.)	Remarks
			A. Active toxin			
1	1·36	0·15	Rt. uncut; peripheral	210	252 (23); 104 (41); 190 (55); 132 (63)	—
2	2·6	0·2	Lt. cut; central end	260	215 (43); 90 (45)	—
3	1·1	0·25–0·5	Lt. cut; central end	294	162 (24)	—
4	1·6	0·5	Lt. uncut; peripheral	396	180 (23)	—
5	0·7	0·5	Lt. cut; central end	264	122 (22); 94 (27)	—
6	1·36	0·5	Lt. cut; ganglion nodosum	372	192 (23); 108 (23·5)	—
7	—	0·7	Lt. cut; central end	280	272 (17); 100 (22)	See Fig. 1
8	2·5	0·75	Rt. cut; central end	260	250 (20); 127 (46); 88 (47)	—
			B. Controls: toxin neutralized by an excess of antitoxin			
9	1·36	0·5	Rt. uncut; centripetal	356	396 (96)	Died of unknown cause on 7th day
10	1·36	0·5	Rt. uncut; centripetal	240	272 (72); 345 (96); 309 (235)	Survived 18 days
11	2	0·5	Rt. uncut; centripetal	309	312 (96); 344 (235)	Survived 18 days
			C. Other controls (active toxin)			
12	2·2	0·9 (+ink)	Subpericardial	292	252 (48)	Injection-site controlled at autopsy (72 hr.) indian ink found between parietal pericardium and ventricle
13	1·1	1	Subpericardial	280	355 (48)	—
14	2·5	0·375	Carotid sinus region	326	307 (70)	Severe torticollis
15	0·9	0·15	Intravenous	320	340 (120); 330 (241)	No symptoms after 10 days
16	0·9	0·25	Intravenous	300	290 (120); 285 (241)	No symptoms after 10 days

Cardiac arrhythmia was always associated with the slowing. In the final stages of the condition complete ventricular stoppage occurred for brief periods (3–4 sec.) when the rabbits would show signs of distress and start struggling. The animal's condition may then worsen rapidly and unless active preventive measures are taken, death from heart failure occurs on the second or third day. A few experiments, not listed in Table 1, with doses of toxin less than 0·15 mg. also resulted in a fatal issue.

Owing to a slight, inevitable, leakage of toxin out of the injected nerve into the surrounding muscles of the neck there was, in nearly all these experiments, a progressive torticollis towards the injected side. But this does not appear to be the initiating cause of the bradycardia because, in other experiments in which severe torticollis followed the injection of 0·1 mg. of toxin into the carotid sheath and of 0·375 mg. into the carotid sinus region there was no bradycardia after 3–4 days, and, conversely, bradycardia without torticollis was produced by the abdominal types of injections (q.v.).

In addition to these symptoms, the peripheral injections into the uncut vagus produced a laryngeal stridor which was sometimes related to the inspiratory, sometimes to the expiratory, phase of respiration. This stridor was audible without the aid of a stethoscope but stethoscopic auscultation revealed that it was loudest over the larynx. It appeared to be due to tetanic spasms of various muscles in the larynx, but no detailed analysis of this effect was made because in the majority of subsequent experiments central-end injections were performed. The results of a few injections into the cut peripheral end of the vagus are at present equivocal and will not be considered here. Symptoms of general tetanus were not seen in any of these experiments.

Specificity of the reaction. That these effects are specifically produced by the toxin is shown in the control experiments in Table 1B, in which 0·5 mg. of toxin, neutralized immediately before the injection with an excess of tetanus antitoxin, was injected into the intact vagus on one side. There was no slowing of the heart and two of the animals survived for 18 days; the other died of an unknown cause on the seventh day, but without bradycardia. The injection of larger doses of toxin (0·9 and 1 mg.) mixed with indian ink into the heart itself, between the perietal pericardium and the ventricular muscle failed to produce bradycardia after 48 hr., when the animals were killed for autopsy.

Two rabbits which received 0·17 and 0·28 mg. of toxin/kg. intravenously survived for 10 days without symptoms of tetanus or any alteration in heart rate (Table 1C).

Paravagal injections in the abdomen

Perioesophageal injections. Cardiac slowing and irregularity was produced regularly by this type of injection (Table 2) with doses of toxin ranging from 0·3 to 1 mg. but the latent period before its onset was somewhat longer than with the injections into the cervical vagus. Again, and in contrast with the

first type of injection, it must be stressed that great care was taken to avoid any mechanical injury to the vagi by contact with the needle. The effect was produced by leaving the uninjured vagi bathed in the solution of toxin (mixed with indian ink in 1 experiment). Five of the animals died of heart failure; the rest were used for experiments. There were no symptoms of generalized tetanus in any of these animals. Injections of boiled toxin, saline, or of 1 mg. of toxin neutralized, just before the injection, with an excess of tetanus antitoxin, failed to produce either bradycardia or death (Table 2B).

TABLE 2. Bradycardia after paravagal injections of tetanus toxin, between the oesophagus and its peritoneal covering in the abdomen

Rabbit no.	Wt. (kg.)	Dose of toxin (mg.)	Resting heart rate (beats/min.) Initial	After injection (hr.)	Remarks
			A. Active toxin		
1	0·9	0·3	293	300 (21); 108* (45)	—
2	1·7	0·45	269	265 (21); 276 (46); 72* (74)	—
3	1·4	0·5	231	226 (50); 234 (93); 209 (120)	Died 120–168 hr.
4	1·25	0·5	240	310 (26)	Died overnight (30–46 hr.)
5	1·5	0·5	246	316 (25); 138† (46)	Died 48 hr.
6	3	0·5	429	284 (48); 272 (76); 127 (96)	—
7	0·9	0·55	332	240 (45); 218† (51)	Died overnight (51–67 hr.)
8	1·4	0·75 (+ink)	312	94† (17)	Died at 18·5 hr.
9	2·3	1	312	163† (42); 84* (46)	—
10	2·6	1	213	288 (27); 202 (44); 134* (72)	—
			B. Controls		
1	1·25	0·5 (boiled)	240	246 (72); 288 (144)	Killed on 6th day; no slowing
2	—	0·5 (boiled)	168	252 (48)	Killed 48 hr.; no slowing
3	0·9	Saline (+ink)	209	240 (72)	Killed 72 hr.; no slowing
4	0·45	1 mg. + excess antitoxin	336	342 (17 days); 435 (20 days); 390 (31 days)	Survived 31 days
5	0·7	1 mg. + excess antitoxin	390	312 (14 days)	Died of unknown cause 17 days later
6	1·6	1 mg. + excess antitoxin	417	318 (17 days); 258 (20 days); 306 (31 days)	Survived 31 days

* Rhythm irregular. † Very irregular.

Anterior wall of the stomach. Bradycardia and death followed the injections of toxin by this route (see Table 3). The injections were performed, again, without mechanical contact with the branches of the left vagus. Symptoms of general tetanus were absent.

TABLE 3. Bradycardia after subperitoneal injections of tetanus toxin along the lesser curvature and anterior wall of the stomach

Rabbit no.	Wt. (kg.)	Dose of toxin (mg.)	Resting heart rate (beats/min.) Initial	After injection (hr.)	Remarks
1	—	0·5 (+ink)	—	228 (3); 248 (23); 96* (46)	—
2	1·4	0·5 (+ink)	—	238 (2); 109* (45); 147 (70)	—
3†	1·1	0·75 (+ink)	302	216 (24); 96* (27)	Died 28·5 hr.
4	1	1	252	125* (21)	Died 36 hr.
5	1·4	1 (+ink)	152	73* (45)	Died 46 hr.

* Irregular.
† This animal had both abdominal vagi cut aseptically 4 days before injecting toxin.

Analysis of the effect

Removal of bradycardia by vagotomy. To facilitate the subsequent performance of vagotomy without anaesthesia, a very fine sterile silk thread was looped carefully round the intact vagi in the neck at the time of the operation for injection. The ends of this loop were knotted together and brought out through a small hole between the skin-sutures in the midline. The free end of the thread was twisted round a short piece of matchstick which was stuck down to the skin with collodion. Loops were prepared round both vagi, except in the experiments on central-end injections in which it was only necessary to prepare one loop round the remaining intact vagus on the side opposite to the injection. When the time came for vagotomy, the matchsticks were cut away from the skin and the loops were pulled out briskly, slicing through the vagi, the whole procedure being quite painless to the animal, as had been shown previously by Pavlov (1910). The vagi were divided after the development of cardiac slowing and at a time when the death of the animal appeared imminent within the next few hours. In all cases, the vagotomy removed the bradycardia (Table 4), indicating that this had been vagal in origin. One of these animals was allowed

TABLE 4

Rabbit no.	Type of toxin injection	Initial heart rate (beats/min.)	Details and time (in hr. after administration of toxin) of vagotomy or of other procedures	Heart rate (beats/min.) Before	Immediately after
	A. Abolition by vagotomy of the bradycardia produced by vagal tetanus				
1	Lt. (cut) central end	264	Rt. cut at 27 hr.	94	269
2	Lt. (cut) central end	294	Rt. cut at 29 hr.	198	432
3	Lt. uncut	396	Both cut at 23 hr.	180	321
4	Paravagal (oesophageal)	429	Both cut at 100 hr.	150	307
	B. Temporary removal of bradycardia by local anaesthesia (5% procaine) of the vagi in the neck				
5	Rt. (cut) central end	260	Procaine (1 c.c.) at 47 hr.	108	393
6	Paravagal (oesophageal)	429	Procaine (0·5 c.c.) at 97 hr.	117	312

to survive after the vagotomy. Its heart rate immediately after vagotomy was 307/min. Twenty-six hours later it was 306/min. and after 43 hr. it was 270/min. and regular. It died 2 hr. later (45 hr. after vagotomy), with gross ulcerations in its stomach. Gastric erosions have been found regularly at autopsy after all 3 types of toxin injection. It is hoped to publish a study of their pathology elsewhere.

Exps. 1 and 2 in Table 4 A deserve especial consideration. In them the toxin was injected upwards into the central end of one cut vagus. The bradycardia was removed by cutting the remaining intact vagus on the opposite side, showing that the slowing was produced entirely by an overactivity, of central origin, of that vagus. Hence, the centripetal injection of tetanus toxin into the vagus nerve on one side, produces a reflex activation of the other vagus.

Effect of blocking the vagus trunk with a local anaesthetic. Local anaesthesia of the remaining intact vagus (or vagi) with 0·5–1 c.c. of a 5 % solution of procaine, injected into the tissues surrounding the vagus in the neck, was, like vagotomy, effective in removing the bradycardia (Table 4B), but this action was not permanent and the bradycardia returned as the anaesthesia wore off.

Atropine. The cardiac slowing of vagal tetanus could be abolished by a peripheral block of the vagi with atropine. However, it is well known that atropine is inactivated fairly rapidly by the serum and tissues of rabbits (Bernheim & Bernheim, 1938). For that reason it could not be expected to have any long-lasting effect on the bradycardia. It did, however, have a considerable immediate effect in raising the heart rate, as shown in all the experiments in Table 5 and in Fig. 1. In Exp. 1 it was necessary to repeat the dose of atropine (0·6 mg./kg.) at 4 hr. intervals because of the transitory nature of its action.

TABLE 5. Temporary removal by atropine of bradycardia produced by tetanus toxin

Rabbit no.	Initial heart rate (beat/min.)	Dose of atropine and time of administration (hr. after the injection of toxin)	Heart rate (beat/min.) Before the atropine	Heart rate (beat/min.) Immediately after
1*	210	0·6 mg./kg. at 67 hr.	168	288
		0·6 mg./kg. at 71 hr.	158	280
		0·6 mg./kg. at 75 hr.	169	276
2	294	1·1 mg./kg. at 45 hr.	108	194
3	270	3 mg./kg. at 74 hr.	72	228
4*	280	1 mg. at 25 hr.	140	250

* Toxin was injected into the cervical vagus in these 2 animals; in the others, paravagally in the abdomen (oesophageal type of injection).

Nembutal. In the early experiments, an attempt was made to obtain a tracing of the heart rate by recording the carotid blood pressure under nembutal anaesthesia. This attempt was foiled because the bradycardia disappeared after the administration of nembutal intravenously (Table 6). For that reason, it was decided, subsequently, to record the heart rate electrically in the intact, unanaesthetized, animals. The effect of nembutal on the heart rate in normal

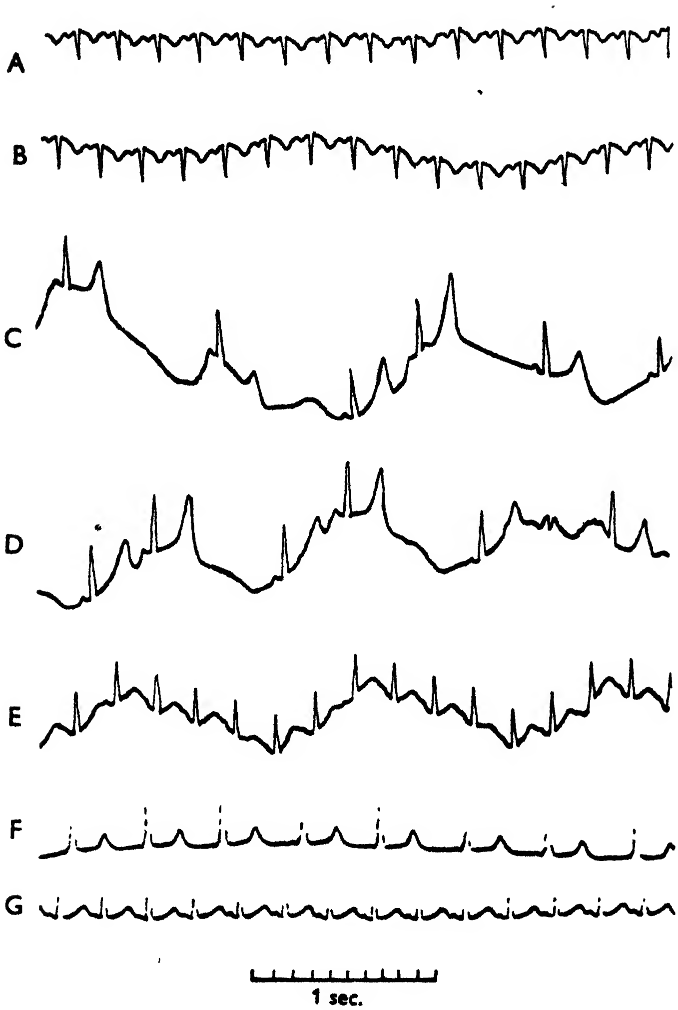


Fig. 1. Bradycardia produced by vagal injections of tetanus toxin (anteroposterior chest lead). A, Initial heart rate (280/min.) after a rest period of 30 min. B, 12 min. after A and 7 min. after the induction of anaesthesia with 58 mg. nembutal i.v.i. (heart rate 274/min.). 0·7 mg. of tetanus toxin was then injected into the central end of the left vagus, 3 in. of the peripheral end being avulsed at the same time. Next day (*leads accidentally reversed*): C and D, 22·5 hr. after the injection: heart rate now very slow (96/min. in C and 109/min. in D) and irregular. Large waves due to respiration but heart beats clearly discernible, superimposed on these. The changes in amplitude of the *R* and *T* waves (*T* is not inverted), and in the duration of the *RT* interval are probably incidental to the bradycardia, since they disappear 4·5 sec. later in E (q.v.). 0·5 sec. after the end of D anaesthesia was induced with 58 mg. nembutal i.v.i. and E starting 4 sec. later, shows the immediate return to the animal's original heart rate (281/min.). F, 140 min. later; the effect of nembutal is wearing off (heart rate 140/min.). 1 mg. of atropine was then administered intravenously and G, started 10 sec. later (heart rate rose to 250/min.). 1 mV = 12·5 mm.

rabbits is negligible (Table 6 B), except in occasional animals which had a heart rate below the average in this series, which was 275 beats/min. On the other hand, in the animals with vagal tetanus, nembutal restored the heart rate to its initial value or higher. This action appears to be a central one since, in the dose in which it was used in these experiments (26 mg./kg.), nembutal was found not to abolish the effect of stimulation of the peripheral end of the vagus nerve.

TABLE 6

Rabbit no.	Initial heart rate before injection of toxin (beat/min.)	Time of administration of nembutal in hr. after the injection of toxin	Heart rate (beat/min.) Before the nembutal	Heart rate (beat/min.) Immediately after
A. Temporary removal by intravenous nembutal (approx. 26 mg./kg.) anaesthesia of the bradycardia produced by tetanus toxin				
1	280	0 hr.	280	274
		23 hr.	102	281
2	312	46 hr.	84	294
3	213	72 hr.	134	345
4	—	50 hr.	120	276
B. Controls: effect of nembutal (approx. 26 mg./kg.) on normal rabbits				
1	—	—	280	274
2	—	—	279	265–330
3	—	—	287	286
4	—	—	180	293
5	—	—	294	318

DISCUSSION

The results show that vagal or paravagal injections of tetanus toxin produce cardiac slowing, after a latent period which is characteristic of tetanus intoxications. Analysis of the effect suggests that it is another example of 'local tetanus', in this case of the cardio-inhibitory centre. This is shown most clearly in those experiments in which the toxin was injected into the central end, i.e. on the afferent side, of one vagus. The cardiac slowing, in experiments of this kind, is due entirely to efferent over-activity of the opposite vagus. The repetitive activity which initiates this type of reflex tetanus could originate either in the sensory nerve fibres on the afferent side, or in the nerve centre itself. However, it is known that the injection of toxin into a pure sensory nerve, if sufficiently remote from the spinal cord, does not elicit reflex tetanus (Fletcher, 1903; Fröhlich & Meyer, 1916). On the other hand, if, as in our experiments, the toxin is injected sufficiently close to the C.N.S., for example into the dorsal roots (Fletcher, 1903), 'tetanus dolorosus' is produced. Fletcher's analysis of this type of 'sensory' tetanus, suggests, again, that the toxin does not act so much on the sensory nerve fibres themselves (or their dorsal root ganglia), as on the nerve centres in the cord with which these fibres are connected. In fact, once 'tetanus dolorosus' had developed, the dorsal roots could be sectioned close to the spinal cord without altering the syndrome.

The centripetal injection of the vagus in the neck forces a certain amount of toxin into the spaces between the nerve fibres and under the perineurium. The solution of toxin can be seen travelling upwards, at the time of the injection, probably in these spaces. Possibly it is directed to the brain stem in this way; this would present no serious anatomical problem. It was, however, surprising to find, when the toxin was placed in the vicinity of the vagi on the anterior wall of the stomach and oesophagus, that the characteristic reflex bradycardia was produced. This appears to indicate some sort of transport of the toxin from those regions to the vagal centre in the medulla. A similar transport, restricted to a single nerve-centre in the spinal cord, from an injection of tetanus toxin into the 'peripheral field' of that centre, has already been reported by Acheson, Ratnoff & Schoenbach (1942). In view of the absence of symptoms of generalized tetanus, the transport does not appear to be blood-borne but possibly intraneural. We have no evidence as to whether this transport would take place within the axons themselves or in the endoneurial spaces. In either case it would appear to be 'against the current'. For the contents of nerve axons are believed to be under pressure from the nerve-cell body and therefore constantly moving in a centrifugal direction (Young, 1944). Similarly, Weiss, Wang, Taylor & Edds (1945) have described a resting flow, distally, of the fluid in the endoneurial spaces. These authors performed very careful experiments with radioactive materials, but their results were obtained mostly within a few hours of nembutal anaesthesia, when presumably the animals were relatively immobile. However, Vishnevsky (1928) showed that methylene blue injected into the sciatic nerve moves centripetally if the limb is actively exercised. Moreover, and this may have a bearing on the pathology of tetanus, Ulyanov (1929) has shown that carmine, injected into the muscles of the calf, is driven up the sciatic nerve in the thigh when the calf-muscles are stimulated electrically.

SUMMARY

1. The injection of tetanus toxin into the central end of one vagus nerve produces, in rabbits, cardiac slowing and arrhythmia. This localized symptom appears after a latent period of 22–43 hr. and terminates in death from heart failure within a few hours, unless the remaining intact vagus is cut.

2. The same effect could be produced by injecting the toxin (*a*) into an uncut vagus nerve, or (*b*) subperitoneally, in the vicinity of the vagus, either in its oesophageal portion or on the anterior wall of the stomach.

3. The reaction is specific and could, in all three types of injection, be prevented by tetanus antitoxin.

4. The cardiac slowing is due to an overactivity of the vagus. It is removed by vagotomy or by local anaesthesia of the vagi in the neck. It can also be abolished for a time (*a*) by a peripheral block of the vagi with atropine, and (*b*) by depression of the vagus centre with nembutal in anaesthetic doses.

5. The analysis of this effect shows that the bradycardia is probably due to a central action of tetanus toxin on the cardioinhibitory centre.

We are indebted to Prof. G. Payling Wright for providing us with the toxin, and to Miss Jean Barrett for helping us with the experiments, which were subsidized by a grant from the Central Research Fund of London University.

REFERENCES

Acheson, G. H., Ratnoff, O. D. & Schoenbach, E. B. (1942). *J. exp. Med.* **75**, 465.

Ambache, N., Morgan, R. S. & Payling Wright, G. (1948). *J. Physiol.* **107**, 45.

Bernheim, F. & Bernheim, M. L. C. (1938). *J. Pharmacol.* **64**, 209.

Courtois-Suffit, M. & Giroux, R. (1918). *The Abnormal Forms of Tetanus.* (Translated by Bruce and Golla.). London: University of London Press.

Dean, H. R. (1917). *Lancet*, **1**, 673.

Fletcher, W. M. (1903). *Brain*, **26**, 383.

Fröhlich, A. & Meyer, H. H. (1916). *Arch. exp. Path. Pharmak.* **79**, [illegible].

Harvey, A. M. (1939). *J. Physiol.* **96**, 348.

Meyer, H. & Ransom, F. (1903). *Proc. Roy. Soc.* B, **72**, 26.

Pavlov, I. P. (1910). *The work of the Digestive Glands*, p. 53–54. London: Chas. Griffin & Co., Ltd.

Ulyanov, P. N. (1929). *Z. ges. exp. Med.* **64**, 78.

Vishnevsky, A. S. (1928). *Z. ges. exp. Med.* **61**, 107.

Weiss, P., Wang, H., Taylor, A. C. & Edds, M. V. (1945). *Amer. J. Physiol.* **143**, 521.

Young, J. Z. (1944). *Nature, Lond.*, **153**, 333.

J. Physiol. (1949) 108, 197–202 612.398.145:612.46

EFFECT OF THE PROTEIN CONTENT OF THE DIET ON THE GLOMERULAR FILTRATION RATE OF YOUNG AND ADULT RATS

By S. E. DICKER

From the Department of Pharmacology, University of Bristol

(*Received* 5 *April* 1948)

It has been shown (Dicker & Heller, 1945) that the kidney function of adult rats, fed on a standard diet, resembled that of the dog, i.e. the rate of glomerular filtration in rats is independent of that of urine flow. In rats fed on a protein-deficient vegetable diet, however, and suffering from severe hypoproteinaemia, the glomerular filtration rate was found to vary with the urine flow (Dicker, Heller & Hewer, 1946). An investigation of the kidney function of normal rats on diets containing different amounts of protein and thus producing different plasma-protein concentrations seemed therefore of interest.

METHODS

Experimental animals. Twenty-nine young rats with weights varying from 101 to 150 g. and ninety-one adult rats with weights from 260 to 350 g. were used.

Diets. The animals were fed on different types of diet, yielding comparable amounts of calories but containing varying amounts of protein.

Diet I (D. I) had the following composition: wheat offal 19·2%, ground wheat 19·2%, finely ground oats 19·2%, ground maize 9·5%, ground barley 9·5%, white fish meal 4·8%, meat and bone meal 9·5%, dried skimmed milk 7·0%, dried yeast 1·3%, salt 0·4% and cod-liver oil 0·4%. 100 g. of this food yielded 305·8 calories. Diet II (D. II) differed from D. I by its higher content of dried skimmed milk, viz. 14% instead of 7%. Its composition was so adjusted as to have the same calorific value as D. I. Diet III (D. III) had the following composition: casein 18%, starch 53%, dried yeast 15·0%, ground-nut oil 8·0%, cod-liver oil 2·0%, salt mixture 4·0%. 100 g. of this diet yielded 303·0 calories. Diet IV (D. IV) differed from D. III by its higher content of casein, viz. 25·0% instead of 18%. Litter-mates were fed on diet D. II until they reached the required weight (101–150 g.). Series of adult rats were fed on the different diets described for 9 weeks.

Experimental procedures for the determination of inulin clearances. The routine procedure conformed on the whole with that described previously (Dicker & Heller, 1945) but differed in the following respect: 1·0 ml./100 g. body weight of a 5% solution of inulin in physiological saline was injected intramuscularly, followed immediately by the administration of 5% of body weight of water by stomach tube. Fifty minutes after water administration the bladder was emptied and the urine-collecting period started; the latter varied between 10 and 15 min. according to the urine flow of the rat. The equilibrium reached 50 min. after the injection was satisfactory: the level of inulin in the plasma did not vary significantly during the period of urine collection. Immediately after the

end of the collecting period, the rats were anaesthetized and blood, obtained from the carotid and jugular vessels, was mixed with heparin.

Analytical methods. Inulin in plasma and urine were determined by the method of Smith, Goldring & Chasis (1938). The inulin used was that of Hopkin and Williams Ltd. Plasma-protein concentrations were estimated by the copper sulphate method for measuring specific gravities (Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald, 1945); Hoch & Marrack's (1945) formula was used for the calculation of the concentration of plasma protein (Dicker, 1948).

Methods of calculation. To permit comparison with previous results (Dicker & Heller, 1945) inulin clearances (=rate of glomerular filtration=G.F.R.) were expressed in ml./100 g. body weight/min.

Statistical treatment of results. Results are given as means and standard error of the mean. The correlation coefficient 'r' was calculated according to Mainland (1938); the probability (P) for 'r' was obtained from the tables of Fisher & Yates (1943). The standard error of 'r' was calculated as $= \pm(1-r^2)/\sqrt{n}$. The coefficient of regression (b) and the regression lines were calculated according to Bradford Hill (1942); the standard error of 'b' was calculated as S.E. $= \pm\sigma/r \times b$ (Pütter, 1929).

RESULTS

In spite of the difference in the composition of the diets given to the rats, they all looked healthy; the increase of weight in adult rats, however, though significant, was not the same in each series; it was least marked in rats fed on diet D. I, in which the average increase of weight in 62 days amounted to 24·0% of the initial weight as compared with 60·0% in rats fed for the same period on diet D. IV (Table 1). Differences in the concentration of plasma

TABLE 1. Effects of protein content of the diet on kidney weight and glomerular filtration rate

	Young rats (29)	Adult rats (21)	Adult rats (25)	Adult rats (22)	Adult rats (23)
Type of diet	D. II	D. I	D. II	D. III	D. IV
Body wt. (g.)	101·0–137·0	265·0–325·0	270·0–345·0	265·0–350·0	260·0–330·0
Increase of wt. in 9 weeks (%)	—	24·0	30·0	45·0	60
Plasma proteins (g./100 ml.)	5·23±0·087	5·84±0·113	6·44±0·047	6·83±0·026	7·33±0·026
Kidney wt. (g.)	1·42±0·082	2·05±0·082	2·08±0·057	2·21±0·085	2·48±0·069
Kidney wt./100 g. body wt.	1·24±0·048	0·75±0·024	0·79±0·052	0·82±0·022	0·88±0·059
G.F.R. (ml./100 g./min.)	Correlated with urine flow			0·43±0·009	0·76±0·335
Coeff. of regression between G.F.R. and urine flow ('b')	+10·0 ±0·54	+8·7 ±0·21	+3·9 ±0·83	−1·4 ±1·09	−0·9 ±2·88

The figures for plasma proteins, kidney weight, kidney weight/100 g. body weight and G.F.R. are mean results with their standard error. 'b' = coefficient of regression and standard error. Number of experiments in parentheses.

proteins, in the kidney weights and in the kidney weight/100 g. body weight ratio of each series were also noticed. Table 1 shows that in young and adult rats kidney weight varied directly with the concentration of the plasma proteins, and that in adult rats the kidney weight/100 g. body weight varied with the type of diet and thus with the plasma-protein concentration.

Results of inulin clearances estimations are given in Fig. 1. They show that in young rats (body weight 101·0–150·0 g.) and in adult rats fed on diets with a low protein content (D. I and D. II), the rate of glomerular filtration was correlated with the urine flow: $b = +10{\cdot}0$, s.e. $\pm 0{\cdot}54$ and $b = +8{\cdot}7$, s.e. $\pm 0{\cdot}41$, respectively. The correlation between g.f.r. and urine flow was still significant

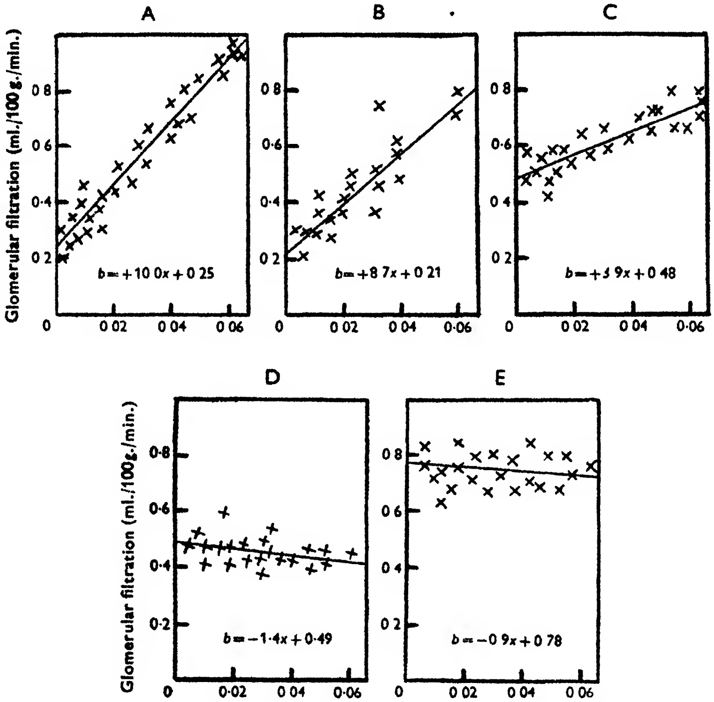


Fig. 1. Effect of protein content of the diet on the glomerular filtration rate of young and adult rats during a water diuresis. A, young rats fed on a diet containing 14% dried skimmed milk; B, adult rats fed on a diet containing 7% dried skimmed milk; C, adult rats fed on a diet containing 14% dried skimmed milk; D, adult rats fed on a diet containing 18% casein; and E, adult rats fed on a diet containing 25% casein. The differences in the slope of the regression line (see also equations) will be noted.

in rats fed on diet D. II: $b = 3{\cdot}9$, s.e. $\pm 0{\cdot}83$. But in rats fed on a diet containing 18% of casein (D. III) the rate of glomerular filtration was independent of that of urine flow (Table 1). The mean value for g.f.r. amounted to $0{\cdot}43 \pm 0{\cdot}009$ (s.e. of mean of twenty-two observations) ml./100 g. body weight/min., a figure that compares with previous results obtained with adult rats fed with the same type of diet (Dicker & Heller, 1945). By increasing the amount of protein in the food (D. IV) and concurrently that in the plasma, the rate of glomerular filtration was significantly increased but remained independent of the rate of

urine flow (Table 1). Its mean value was $0{\cdot}76 \pm 0{\cdot}335$ (23) ml./100 g. body weight/min., a value comparable with that found by Lippman (1947), and by Friedman, Polley & Friedman (1947).

DISCUSSION

Recent work on kidney function in rats conveys the impression of a lack of agreement with reference to (*a*) the relation between the rate of glomerular filtration and that of urine flow, and (*b*) the mean value of the inulin clearances (=glomerular filtration rate). Some workers (Dicker & Heller, 1945; Friedman *et al.* 1947; Lippman, 1947) found that the inulin clearances in adult rats were independent of the rate of urine flow, thus resembling in this respect adult man and the dog (Smith, 1937); others (Braun Menendez & Chiodi, 1946; Friedman, 1947) claimed that the inulin clearances varied directly with the urine flow, as in rabbits (Kaplan & Smith, 1935; Dicker & Heller, 1945; Forster, 1947). Further confusion arose from the fact that some workers (Dicker & Heller, 1945; Friedman *et al.* 1947) related the mean values of the inulin clearance to body weight, others (Braun Menendez & Chiodi, 1946) to body surface and others again (Lippman, 1947) to kidney weight.

Kidney weight is accepted to be related to body weight and surface area and is usually calculated from body weight, hence the choice of the parameters would seem to be immaterial. But, as it could be shown that the kidney weight/100 g. body weight varied (*a*) with the age of the rat and (*b*) with the type of diet given to the animal, it follows that none of the proposed parameters (body weight, kidney weight or surface) can be relied upon unless the type of experimental animal and the diet have been standardized.

The importance of standardization is also borne out by the lack of agreement in the results of the inulin clearances estimated by different workers. Braun Menendez & Chiodi (1946) used eighty-four rats with a weight varying from 121·0 to 430·0 g., thirty of which weighed less than 165·0 g.; these authors found that there was a very close correlation between the rate of glomerular filtration and that of urine flow ($r = +0{\cdot}87$). If the series of Braun Menendez & Chiodi (1946) were divided into two groups, one of rats with a weight varying from 121·0 to 200·0 g. and another of rats with a body weight from 201·0 to 430·0 g., it could be calculated from their data that the correlation between the rate of glomerular filtration and urine flow in the first group was highly significant, but only just significant in the second ($r = +0{\cdot}33$; $P < 0{\cdot}05 > 0{\cdot}02$). This suggests that the first group compares with the 'young rats' of the present series and the second group with the more adult rats of the present series which were fed on a diet which had a low protein content.

A last point to be considered is the rate of the glomerular filtration. Shannon (1942) has shown that the administration to dogs of a saline solution (85 mmol. NaCl) increased significantly the rate of the glomerular filtration; the

same observation has been made in rats (Dicker, 1946). Furthermore, Ayer, Schiess & Pitts (1947) have shown that in dogs fed on a meat diet the rate of glomerular filtration could increase by nearly 100% when compared with dogs fed on a carbohydrate diet. A similar finding was made in rats; it could be shown that by varying the amount of casein in the diet from 18·0 to 25·0%, the mean inulin clearance (=glomerular filtration rate) could be increased from 0·43 to 0·76 ml./100 g. body weight/min.

It is thus clear that provided the animal material is standardized, with reference to age and to diet, good agreement will be found in estimating the kidney function in rats.

SUMMARY

1. Inulin clearances (=glomerular filtration rate) were estimated (*a*) in young rats with weights ranging from 101·0 to 150·0 g., (*b*) in adult rats with weights varying from 260·0 to 350·0 g.

2. The plasma-protein concentration of the adult rats varied with the amount of protein in the diet.

3. In young and in adult rats with a plasma-protein concentration below 6·80 g./100 ml., the rate of glomerular filtration was correlated with that of the urine flow.

4. In adult rats fed on a diet containing at least 18% casein, and having a plasma-protein concentration above 6·80 g./100 ml., the rate of glomerular filtration was independent of that of the urine flow.

5. High concentrations of protein in plasma were accompanied by higher mean rates of glomerular filtration.

This work was carried out while I was holding a Beit Memorial Fellowship. The expenses of this investigation were partly defrayed by a grant from the Colston Research Committee, whose help is gratefully acknowledged. The author expresses his appreciation for the technical assistance of Miss P. A. Ashby.

REFERENCES

Ayer, J. L., Schiess, W. A. & Pitts, R. F. (1947). *Amer. J. Physiol.* **151**, 168.

Bradford Hill, A. (1942). *Principles of Medical Statistics*, 3rd ed. p. 103. London: The Lancet Ltd.

Braun Menendez, E. & Chiodi, H. (1946). *Rev. Soc. argent. Biol.* **22**, 314.

Dicker, S. E. (1946). *Brit. J. Pharm. Chem.* **1**, 194.

Dicker, S. E. (1948). *J. Physiol.* **107**, 11 *P.*

Dicker, S. E. & Heller, H. (1945). *J. Physiol.* **103**, 449.

Dicker, S. E., Heller, H. & Hewer, T. F. (1946). *J. exp. Biol.* **27**, 158.

Fisher, R. A. & Yates, F. (1943). *Statistical Tables for Biological, Agricultural and Medical Research.* Edinburgh and London: Oliver and Boyd.

Forster, R. P. (1947). *Amer. J. Physiol.* **150**, 523.

Friedman, M. (1947). *Amer. J. Physiol.* **148**, 387.

Friedman, S. M., Polley, J. R. & Friedman, C. L. (1947). *Amer. J. Physiol.* **150**, 340.

Hoch, H. & Marrack, J. (1945). *Brit. med. J.* **2**, 151.

Kaplan, B. I. & Smith, H. W. (1935). *Amer. J. Physiol.* **113**, 354.

Lippman, R. W. (1947). *Amer. J. Physiol.* **151**, 211.

Mainland, D. (1938). *The Treatment of Clinical and Laboratory Data.* Edinburgh and London: Oliver and Boyd.

Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B. & Archibald, R. M. (1945). *Copper Sulphate Method for Measuring Specific Gravities of Whole Blood and Plasma.* New York: Josiah Macy, Jr. Foundation.

Pütter, A. (1929). *Die Auswertung zahlenmässiger Beobachtungen in der Biologie.* Berlin and Leipzig: Walter De Gruyter and Co.

Shannon, J. A. (1942). *J. exp. Med.* **76**, 371.

Smith, H. W. (1937). *The Physiology of the Kidney.* Oxford University Press.

Smith, H. W., Goldring, W. & Chasis, H. (1938). *J. clin. Invest.* **17**, 263.

J. Physiol. (1949) 108, 203–217 612.844.4/7

THE PENETRATION OF SOME ELECTROLYTES AND NON-ELECTROLYTES INTO THE AQUEOUS HUMOUR AND VITREOUS BODY OF THE CAT

By H. DAVSON, W. S. DUKE-ELDER, D. M. MAURICE, E. J. ROSS and A. M. WOODIN

From the Department of Physiology, University College, London

(*Received* 6 *April* 1948)

A comprehensive study of the kinetics of penetration of various substances into the eye should throw some light on the mechanism of formation, and conditions of circulation of the intra-ocular fluids. As a first step in this study it is necessary to know the relative rates at which various substances enter the fluids from the blood, and to attempt to relate these with molecular structure. It is with this aspect that the present paper is concerned; the study is by no means complete, but it is considered that the results so far obtained are of sufficient general interest to justify publication.

THEORETICAL

The application of Fick's law to diffusion through the boundary separating the aqueous humour from the plasma gives the equation

$$dS/dt = kA(C_p - C_{Aq}), \tag{1}$$

where S is the amount of the diffusing substance present in the aqueous humour at any time t; A is the area of the boundary; C_p and C_{Aq} are respectively the concentrations of the substance in plasma and aqueous humour at any time t, and k is a permeability constant. The important assumption must be made that the rate of penetration of the boundary is slow compared with the rate of diffusion in the aqueous humour and plasma. The propriety of this assumption will be discussed later. Equation (1) gives on integration at constant C_p

$$1/t \log \frac{C_p - C_{Aq_0}}{C_p - C_{Aq}} = \frac{1}{2 \cdot 303} kA/V = K_A, \tag{2}$$

where V is the volume of the aqueous humour, and C_{Aq_0} is the initial concentration of the penetrating substance in the aqueous humour.

In the absence of definite knowledge of the ratio A/V, the parameter K_A may be used to indicate the rate of penetration of a substance into the aqueous humour. A similar parameter K_V may be assessed for the rate of penetration into the vitreous body. The direct comparison of K_A and K_V does not, of course, indicate the relative permeabilities of the boundaries separating the aqueous humour and vitreous body from the blood plasma, since A and V are different in the two cases. Equation (2) has been used to describe the results of earlier studies (Davson & Quilliam, 1940; Davson & Duke-Elder, 1948); in the present work, however, a less approximate treatment is required. In the case of a penetrating ion the equilibrium condition is not given by equal concentrations in plasma and aqueous humour, but is described by the Gibbs-Donnan equilibrium such that $C_{Aq}/C_p=r$, where r is greater or less than unity according as the substance considered is a negative or positive ion. We may assume that the kinetics of penetration are represented by

$$\frac{dC_{Aq}}{dt}=k_pC_p-k_aC_{Aq}, \tag{3}$$

at equilibrium we have $dC_{Aq}/dt=0$, whence

$$C_{Aq}/C_p=\frac{k_p}{k_a} \quad \text{or} \quad k_p=rk_a.$$

On integration of (3) at constant C_p we now have

$$k_p=\frac{r}{t}\log\frac{rC_p-C_{Aq0}}{rC_p-C_{Aq}}. \tag{4}$$

With certain non-electrolytes, notably urea, the ratio of C_{Aq}/C_p in vivo is about 0·8. This condition may be treated formally as if it were the result of a larger permeability constant for diffusion out of the eye than that for diffusion in, i.e. equation (4) could be applied. Recently Kinsey & Grant (1942) have suggested an explanation which leads to an identical mathematical formulation; they emphasize that aqueous humour is constantly being drained away and must be replaced by an ultrafiltrate from plasma; if the ultrafiltrate is free from the penetrating substance a steady state is achieved with a value of r less than unity. They set up an equation

$$dS/dt=kA(C_p-C_{Aq})-k'C_{Aq},$$

where k' represents the volume of aqueous humour drained away in unit time. This equation contains the somewhat improbable implication that the penetrating substance is retained completely in the plasma during the ultrafiltration process. The state of affairs has been formulated more generally (Duke-Elder & Davson 1943; Palm 1947):

$$dS/dt=V\frac{dC_{Aq}}{dt}=kA(C_p-C_{Aq})-k'C_{Aq}+nk'C_p, \tag{5}$$

where n is a factor representing the discrepancy between the concentrations of the substance in the plasma and its ultrafiltrate. This equation is identical with (3) with r given by

$$r=\frac{kA+nk'}{kA+k'},$$

so that integrating at constant C_p gives

$$\frac{r}{t}\log\frac{rC_p-C_{Aq_0}}{rC_p-C_{Aq}}=\frac{1}{2\cdot303}\,\frac{kA+nk'}{V}=K', \tag{6}$$

The results to be presented in this paper will be presented as the parameter K'; according to the assumptions at the basis of equation (4) it would be proportional to the permeability constant from plasma to aqueous humour, whilst according to equation (6) it would be proportional to the permeability constant plus an ultrafiltration or drainage factor, nk'. Except in the case of urea and the electrolytes, there is considerable uncertainty regarding the magnitude of r; fortunately, however, the parameter K' is very insensitive to variations in this quantity, so that changes in K' on passing from one molecule or ion to another must be due mainly to changes in permeability.

METHODS

General. The technique was similar in essentials to that described earlier (Davson & Duke-Elder, 1948); a high and relatively constant concentration of a given substance was maintained in the blood of an anaesthetized cat; this was achieved, in general, by a single intravenous injection of an isotonic solution followed by repeated smaller injections in accordance with empirically determined schedules which varied with the substance considered. In the case of radioactive potassium (K^{42}), however, the rate of loss from the blood was so rapid that it was impracticable to maintain a constant high level by this means; consequently the solution was injected by a drip-infusion technique; the concentration of K^{42} rose rapidly during the first few minutes, and then more slowly and approximately linearly during the rest of the experiment. The parameters K_A' and K_V' were calculated by graphical integration. When sugars, creatinine, urea and amino-acids were studied the renal arteries were tied before the injection. The operative technique was essentially that described by Liddell & Sherrington (1929) for isolation of the splanchnic nerve. The eyes and blood samples were removed at appropriate intervals and the aqueous humour, vitreous body and plasma submitted to analysis. With sugars, amino-acids and urea, one eye had to be used as a control (i.e. to give the amount of substance present in the eye at zero time). With the remaining substances this was not necessary, so that two constants could generally be obtained on the same animal.

Chemical. Sugars were determined by the Hagedorn-Jensen (1923) technique on Somogyi (1930) filtrates; thiocyanate by the colorimetric technique of Aldridge (1945) on trichloracetic acid filtrates; creatinine by the Folin (1905) method on tungstic acid filtrates; amino-acids by Danielson's (1933) modification of the Folin-Wu (1919) method on tungstic acid filtrates, and urea by the Conway method (1947).

Radioactive tracers. With the exceptions described below, all the determinations of radioactive tracers were done with the pipette-type counter described earlier (Maurice, 1948) which was supplied with its high tension from a Dynatron Type 200 power unit, the counts being recorded on a Type 200 scale-of-ten scaler preceded by a modified Neher-Pickering circuit. The counter was always washed and dried between samples except when there was a reasonable certainty that the difference in activity was small. Sufficient counts were made to give a standard deviation of

between 1 and 2% in the case of plasma and aqueous humour and of 5% in the case of the vitreous body. Corrections were applied for the background count, the decay of the samples, and the resolution time of the counter. This last correction never rose above 2 or 3%. The voltage and temperature of the counter were noted, but they never varied sufficiently during an experiment to make a correction necessary.

In the case of the first five measurements of Na^{24} activity, the samples were measured into small glass dishes, which were stood on the window of a bell-type counter and located by means of a wax cast fixed by the edge of the window. Quadruplicate 0·2 ml. samples were used, the agreement being that expected from the number of counts taken.

For the first four phosphate determinations the sample, after removal of proteins if necessary, was treated with sodium phosphate solution to give about 1 mg. of precipitate, excess of magnesium and ammonium chlorides was added and the mixture made alkaline with ammonia. The precipitate of magnesium ammonium phosphate was collected by suction on a sintered glass filter, 4 mm. in diameter, sealed into the end of a glass tube. It was found necessary to repeat the precipitation on the first filtrate in order to complete the collection of the phosphate. The collected precipitate was dried, and the 'filter stick' was accurately positioned by a brass frame so that the precipitate was brought into proximity with the window of a bell-type counter. Na^{24} w, used in the form of sodium chloride with an activity of 1 mC./g.; K^{42} as potassium chloride of similar activity. P^{32} was received as a solution of phosphoric acid containing less than 10 mg./ml. of total solids and with an activity greater than 0·3 mC./ml. This was brought to pH 7·4 with NaOH.

RESULTS

The general results are presented in Table 1. The rates of penetration into aqueous humour and vitreous body are represented by the constants K'_A and K'_V, the significance of which has been discussed above. It may be repeated that K'_A and K'_V are not true permeability constants, since they contain an undetermined factor, A/V, the ratio of the area to the volume of the system; K'_A and K'_V are therefore not directly comparable with each other. For convenience, we have included in the table some earlier described results on sugars.

TABLE 1. The rates of penetration of various substances into the aqueous humour (K_A') and vitreous body (K_V'). Standard errors are indicated

Substance	No. of exps.	$100\ K_A'$	$100\ K_V'$	K_A'/K_V'
Thiocyanate	12	46·5 ± 2·3	18·3 ± 1	2·5
Sodium	15	37·6 ± 1·2	6·8 ± 0·3	5·5
Potassium	14	42 – 31	12 – 7·7	3·5–4
Phosphate	6	5·2 ± 0·9	0·33 ± 0·05	16
Monosaccharides	27	32·5 ± 1·2	11·2 ± 0·5	2·9
Sucrose	9	4·7 ± 0·6	0·28 ± 0·05	17
Urea	12	14 ± 0·9	7 ± 1·2	2
Creatinine	8	12·9 ± 1·2	0·79 ± 0·13	16
Glycine	3	15·5 ± 2·3	0·6 ± 0·3	26
Alanine	5	13·3 ± 2·4	1·0 ± 0·5	13

There is little doubt that we are dealing with a selective membrane or system of membranes; in general it would seem that nitrogenous compounds penetrate the intra-ocular fluids slowly in comparison with other substances of similar size. Penetration into the vitreous body, as indicated by the values of K'_V, appears to be generally slower, but this may be due to an unfavourable relationship of area to volume and less efficient circulation. If these were the only

factors concerned however, it would be expected that the ratio K'_A/K'_V for different substances would be fairly constant; this is by no means true, as Table 1 shows.

Of the strong electrolytes it is seen that thiocyanate penetrates most rapidly with a K'_A of 46·5: sodium and potassium have significantly lower values of 37·6 and 35·6 respectively. All values of K'_A and K'_V presented in this paper have been multiplied by 100; this has been indicated in the tables, but, to avoid tiresome repetitions, not in the text.) The monosaccharides with an average K'_A of 32·5 thus penetrate the aqueous humour rather more slowly than the three ions considered above. Sodium and potassium penetrate the vitreous body more slowly than the monosaccharides, whilst thiocyanate enters more rapidly. Phosphate penetrates slowly into the aqueous humour ($K'_A = 5·2$) and very slowly into the vitreous body (K'_V 0·33).

TABLE 2. The simultaneous penetration of sugar and thiocyanate into the eye of the cat

Substance	$100\,K_A'$	$100\,K_V'$
{Thiocyanate	42·5	16·8
{Galactose	26·7	6·5
{Thiocyanate	40	20
{Glucose	27·1	7·8
{Thiocyanate	27·5	16
{Galactose	21·2	4·6

Thiocyanate. Dialysis experiments indicated that only 80 % of the thiocyanate in blood was diffusible through a collodion membrane; presumably the remainder is adsorbed on to protein. A value of 1·05 was taken for r, the equilibrium distribution of diffusible thiocyanate between aqueous humour and plasma on the assumption that it would behave as chloride. The mean value of K'_A is given in Table 1 as $46·5 \pm 2·3$, which compares with a value of $32·5 \pm 1·2$ for the monosaccharides; the direct comparison of the rates of penetration of thiocyanate and sugar into the eyes of the same animal is in good agreement with these values (Table 2). Since very small amounts of this substance can be accurately determined, it was possible to vary the blood concentrations over a comparatively wide range, and thus ascertain whether the constants K'_A and K'_V varied with the concentration gradient; this is generally considered to be a suitable test to distinguish between a simple kinetic phenomenon and a secretory process. In Table 3 the values of K'_A and K'_V are shown for blood concentrations varying from 58 to 9 mg./100 ml. There seems to be no definite drift in the values of K'_A and K'_V as the concentration gradient is lowered.

In earlier experiments on the penetration of sugars, the frozen vitreous body was separated into three parts by sections at right angles to the anterior-posterior axis; the anterior portion was closest to the ciliary body, the posterior part farthest away. It was suggested that, if diffusion from the blood into the vitreous body could occur not only from the ciliary body but also from the

TABLE 3. The values of K_A' and K_V' for the penetration of thiocyanate with decreasing concentrations of this ion in the plasma

Plasma concentration (mg./100 g. H_2O)	100 K_A'	100 K_V'
58	51	19
27·5	47	17·5
23·2	42·5	17·5
21·7	49·5	13
20·4	40·5	20
19·6	52·5	—
17·5	42	20
14·8	60	—
14·8	27·5	16
14·3	46·5	17·5
9	48	23·5

choroid, the middle section would have the lowest concentration of the penetrating substance owing to the unfavourable area-volume relationship pertaining in this region. If, on the other hand, diffusion occurred exclusively from the ciliary body, the posterior portion would have the lowest concentration. With the sugars it was found that the middle sections, on the average, had the lowest concentration at the end of a given period of diffusion. With thiocyanate essentially similar results were obtained, the concentrations in the aqueous humour and the different parts of the vitreous body being as shown in Table 4. One may tentatively conclude from these results that thiocyanate diffuses into the vitreous body from both the choroid and ciliary body, the low concentration in the middle section being due to the unfavourable area-volume relationship prevailing in this region.

TABLE 4. Relative concentrations of thiocyanate in the three sections of the vitreous body after the concentration in the blood had been maintained at a high value for various times

	Concentration			
Time (min.)	Aqueous humour	Anterior	Middle	Posterior
30	100	46	49	46
30	100	55	64	82·5
31	100	47	34	55
34	100	39	28	33
38	100	53	37	64
60	100	58	42	44
60	100	55	37	57
60	100	56	52	80
60	100	68	68	80

Amino-acids. It is doubtful whether the 'equilibrium ratio' of the concentrations of amino-acids in aqueous humour and plasma has any significance owing to the poor specificity of the analytical method; a ratio of 0·75 was somewhat arbitrarily taken by analogy with urea which penetrates the aqueous humour at about the same rate. The mean values for K'_A and K'_V are in the region of 14 and 1 respectively. Insufficient experiments have been carried out

to permit of an accurate estimate of the comparative rates of the two acids, glycine and alanine; certainly any difference is unlikely to be large. In one experiment the rates of penetration of sucrose and alanine into the same eye were determined; the results were:

	K_A'	K_V'
Sucrose	5·1	0·9
Alanine	12·2	1·5

i.e. alanine entered the aqueous humour at about twice the rate of sucrose; from Table 1 it will be seen that this accords fairly well with the relative rates estimated from the means of several animals.

Urea and creatinine. The mean value for r for urea is 0·75; the same figure has been used for creatinine. These substances behave similarly to the amino-acids, in that their rates of penetration into the aqueous humour are of the same order (K'_A for urea 14; K'_A for creatinine 12·9). Urea is differentiated from both the amino-acids and creatinine, however, in having a fairly large rate of penetration into the vitreous body ($K'_V = 7$ compared with 0·8 for creatinine and about 1 for amino-acids). In one experiment sucrose and creatinine were injected into the same animal; the values for K'_A were 13·6 and 7·1 for creatinine and sucrose respectively, whilst the mean values from Table 1 give 12·9 and 4·7.

Sodium. In Table 5 are shown the calculated constants, K'_A and K'_V, for the penetration of Na^{24} into the aqueous humour and vitreous body respectively; since two constants may be determined in each animal, two values are presented. The agreement between the two for any given cat is remarkably good, in fact, surprising, when one considers that the mode of bulk intravenous injection employed in this work introduces an appreciable error in the estimate of the average concentration gradient between plasma and intra-ocular fluid. Thus during the period of the first injection of about 18 ml., which lasted about 1 min., the blood reaching the eye had a very considerably higher concentration of Na^{24} than that circulating through the eye a few minutes later after mixing had occurred with the rest of the blood. This error enters into the determination of the first value of K' but not of the second; experiments in which very brief periods of diffusion into the eye were studied indicated that over a period of half an hour—the usual interval for the first constant—the error should be in the region of 2 units.

TABLE 5. Penetration of Na^{24} into the aqueous humour (K_A') and vitreous body (K_V') of the cat. The suffices refer to the first and second half-hours of diffusion

$100\,K_{A_1}'$	$100\,K_{A_2}'$	$100\,K_{V_1}'$	$100\,K_{V_2}'$
42·7	46·2	7·1	7·5
38·5	—	8·2	6·5
34·1	32·9	5·9	4·1
32·0	29·8	6·2	8·4
36·3	33·5	6·4	6·9
44·3	47·7	8·7	4·8
42·8	34	4·9	8·0

The mean values of K'_A and K'_V are 37·6 and 6·8 respectively. In two animals thiocyanate and Na^{24} were injected at the same time, the mean values of K'_A and K'_V were as follows:

	K_A'	K_V'
Sodium	34·4	6·8
Thiocyanate	43·7	20·7

There is thus no doubt that thiocyanate enters the intra-ocular fluids more rapidly than sodium, the difference being most marked in respect to the vitreous body.

TABLE 6. Relative concentrations of Na^{24} in the three sections of the vitreous body after the concentration in the blood had been maintained at a high value for various times

	Relative concentrations			
Time (min.)	Aqueous humour	Anterior	Middle	Posterior
28	100	44	12	18
60	100	38	29	25
7	100	27	12	6
8	100	24	11	10

As with thiocyanate, an attempt was made to determine the site of entry of sodium into the eye by freezing the vitreous body and submitting three sections to analysis. A striking difference in distribution was observed as Table 6 shows; the order of descending concentration was in nearly all cases

$$\text{Anterior} > \text{Middle} > \text{Posterior}.$$

This strongly suggests that penetration into the eye is predominantly by way of the ciliary body and iris, and that penetration from the choroid and retinal circulation is, in comparison, small. In order to be certain that the differences in concentration were not a factitious result of the freezing process, eyes containing Na^{24} uniformly distributed throughout the vitreous body were similarly sectioned. No consistent difference in concentration between the several parts was observed. The uniform distribution of Na^{24} was obtained either by allowing Na^{24} to achieve equilibrium between blood and eye by waiting some hours after the injection before removal of the eye, or by allowing diffusion equilibrium to take place in the eye after enucleation, by keeping it in the ice-chest for 24 hr.

Potassium. The results with K^{42} were more variable, possibly because potassium is pharmacologically active, the rate of injection of the solutions being sufficiently great to modify the concentration of potassium in the blood. The variability manifested itself particularly in a tendency for the value of K' appropriate to the second half-hour of diffusion to be less than that for the first half-hour, i.e. the rate of penetration of potassium seemed to slacken off with time. The average results were as follows:

K_{A_1}'	K_{A_2}'	K_{V_1}'	K_{V_2}'
40·5±6·3	30·3±4·5	11·8±0·5	7·4±0·4

where the suffices refer to the first and second half-hours of diffusion. The effect is the more pronounced with K'_V. Sectioning and analysis of the frozen vitreous bodies gave the relative concentrations shown in Table 7; the figures are similar to those obtained with sodium and in contrast to those obtained for thiocyanate and the monosaccharides.

TABLE 7. Relative concentrations of K^{42} in the three sections of the vitreous body after the concentration in the blood had been maintained at a high value for various times

	Relative concentrations			
Time (min.)	Aqueous humour	Anterior	Middle	Posterior
30·5	100	43	20	14
31	100	54	20	13
31	100	92	41	16
31	100	49	39	18
38	100	53	23	20
59	100	64	44	23
58	100	71	31	13

Phosphorus. Results with P^{32} must be treated with caution, since although it is injected as inorganic phosphate it apparently distributes itself with the various organic fractions; the effective concentration of phosphate ion may therefore not be indicated by the radioactivity of the solution. The results suggest that phosphate penetrates into the aqueous humour slowly (K'_A 5·2), and into the vitreous body still more slowly ($K'_V = 0{\cdot}33$).

Glycerol. Several attempts were made to measure the rate of penetration of glycerol into the eye fluids; the method of Silberstein, Rappaport & Reifer (1937) was used, but owing to the large blanks it was not very accurate. The results have not been included in Table 1, but the value of K'_A is in the region of 20.

DISCUSSION

The results presented in this paper indicate that the barrier separating the aqueous humour from the blood plasma possesses a selectivity capable of discrimination not only on a basis of molecular size as with the series of sugars, but also on the basis of chemical structure—witness the slow rate of penetration of nitrogenous compounds. The significance of chemical structure is revealed not only with the nitrogenous substances; sorbitol is not greatly different in size and the number of its water-soluble groups from glucose, yet it is claimed that it enters the eye extremely slowly, more slowly than sucrose (Rosner & Bellows, 1939). Again, glycerol has only about half the size of glucose and contains fewer water-soluble groups, yet it penetrates the aqueous humour at only some two-thirds the rate of glucose. Apparently there is a specific chemical grouping in the sugars that permits their ready passage into the eye. The importance of lipoid solubility in penetration into the aqueous humour is revealed by a recent study of Palm (1947), who showed that ethyl alcohol penetrated the rabbit's eye very rapidly ($K'_A = 180$). The barrier between blood

and aqueous humour is a composite entity; in the region of the ciliary body—where we may expect penetration to occur most prominently—the barrier consists of the capillary endothelium, the lamina vitrea, and a double layer of ciliary epithelial cells. A fairly high degree of selectivity may be expected from the last-named structure by analogy with the tubular epithelium of the kidney; molecules would probably penetrate between the epithelial cells or through them, the relative extents to which the two paths were used depending principally on the number of water-soluble groupings in the molecule and also on the presence of specific chemical groupings. With strongly water-soluble substances the intercellular route would be used and the pore size, i.e. the diameter of the intercellular spaces, would determine the upper limit of molecular size consistent with penetration; earlier studies (Weld, Feindel & Davson, 1942) have shown that the upper limit for the saccharides lies between sucrose and raffinose. The high rate of penetration of glucose is not consistent wit' this simple picture, since its molecules are large and lipoid-insoluble. It is thought, however, that cell membranes are specialized to permit the rapid penetration of certain biologically important substances, and it would seem that the high rate of penetration of glucose (and of galactose, xylose and 3-methyl glucose) is an expression of this specialization; glucose is necessary to the metabolism of cells and must therefore be able to penetrate in spite of its lipoid insolubility and large molecular size.

The nitrogenous compounds studied here, creatinine, glycine, alanine and urea, penetrate the aqueous humour at about the same rate. If these substances penetrated only through water-filled pores (e.g. the intercellular spaces), a significant difference in rates of penetration between creatinine (M.W. 112) and urea (M.W. 60) should be observed; and if the pores were small, of a size comparable with that of the penetrating molecules, this difference would be exaggerated (Davson & Danielli, 1943). The fact that the rates of penetration are so close to each other reveals, once again, the inadequacy of any simple pore theory of membrane function. It is interesting that penicillin, a nitrogen-containing substance, penetrates the aqueous humour very slowly indeed; a constant K'_A of about unity may be computed from v. Sallmann and Meyer's figures (1944), i.e. the rate of penetration is only about one-fifth of that of sucrose. Sucrose has a molecular weight of 342 whilst that of penicillin is 256, so once again the importance of chemical structure in determining the ease of penetration is emphasized.

The rapid rates of penetration of the ions Na^+, K^+ and CNS^- could be due to their small size permitting more or less unrestricted diffusion through the intercellular spaces; the higher rate of penetration of the CNS^- ion suggests, however, that the cellular route is also of some importance at least for this ion. The very slow rate of penetration of phosphate might indicate that the size of the intercellular pores was comparable with that of this ion. Phosphate is

a heavily hydrated anion and may have a diameter considerably greater than that to be expected on the basis of its ionic weight alone. The results with this radioactive isotope must, however, be treated with caution owing to the intimate relationship between phosphorus and metabolism generally; the behaviour of the sulphate ion, of comparable size and hydration, would be interesting in this connexion.

We have so far discussed the penetration of the various substances into the aqueous humour; it has been seen that they also penetrate the vitreous body and that the rate of approach to diffusion equilibrium is always slower. It has been emphasized, however, that this need not necessarily indicate smaller permeability constants but could be explained by an unfavourable area-to-volume relationship. Moreover, we must bear in mind that the parameters K'_A and K'_V have been obtained from an equation on the assumption that the rate of penetration of the barrier is small compared with the rate of diffusion in the intra-ocular fluids. In respect of the aqueous humour this assumption appears to be justified, in so far as the substances discussed here are concerned, for the following reasons: (a) The aqueous humour is now known to circulate, in the sense that it is drained away at the angle of the anterior chamber and replaced by new fluid. (b) The values of K'_A for comparatively rapidly penetrating substances like sodium and glucose are not appreciably greater when computed for the first half-hour of diffusion than when computed from the second half-hour. (c) The times of diffusion were generally chosen so that the system was less than half way to equilibrium at the time of removal of the fluid; the error due to a failure of the penetrating substance to mix completely with the aqueous humour becomes more significant the nearer the penetration process is to equilibrium at the time of withdrawal of fluid. These arguments do not necessarily apply to penetration into the vitreous body, however. So far there has been no demonstration of a circulation of fluid through the vitreous body, and it is, moreover, possible that the area-to-volume relationship may not be so favourable for rapid mixing. That complete mixing does not occur during the process of penetration is shown, furthermore, by the studies on the frozen vitreous body described here and elsewhere (Davson & Duke-Elder, 1948). We may therefore expect that the parameter K'_V will be a less accurate measure of the rate of penetration of different substances into the vitreous body than the parameter K'_A of the penetration of substances into the aqueous humour. In general, the factor of inadequate mixing will tend to obscure differences in rates of penetration, so that the computed differences will be rather smaller than the real ones. In view of the quite large differences actually found (the largest value of K'_V is some fifty times greater than the smallest) it would seem that the error involved in the assumption is not sufficiently great to affect any general conclusions from the observed values of the parameter K'_V. It will be quite unjustifiable, however, to argue that, because K'_V is smaller than K'_A, the permeability of the barrier separating vitreous body from plasma

is less than that of the barrier separating the aqueous humour from plasma. Nevertheless, if the factors of different area-to-volume relationships and inadequate mixing were alone the causes of the smaller values of K'_V, the constants K'_A and K'_V would bear a more or less uniform relationship with each other, whereas a study of the ratio K'_A/K'_V in Table 1 indicates a wide variability that can only be due to differences in the permeability of the barriers separating blood on the one hand from aqueous humour and vitreous body on the other. Penetration into the vitreous body may occur from the ciliary body—so that this site of penetration is common to both aqueous humour and vitreous body; from the choroid through the membrane of Bruch, the pigment epithelium and the pars optica retinae; and from the capillaries of the retinal circulation. Studies on the sugars have shown that the monosaccharides may penetrate both from the ciliary body and from the posterior region; such a finding is reasonable in view of the metabolic needs of the retina. The present studies on the electrolytes have shown that sodium and potassium enter predominantly by way of the ciliary body whilst thiocyanate appears to enter from all parts of the periphery of the vitreous body. Thiocyanate is an ion at the extreme end of the lyotropic series; the results of numerous studies agree in assigning to it a high permeability through biological membranes (vide e.g. Davson & Danielli, 1943); it is not surprising, therefore, that it should be able to penetrate all parts of the barrier separating blood from vitreous body. The fact that sodium and potassium apparently do not penetrate the more posterior part of this barrier to any extent fits in with recent findings on the permeability of the cerebral capillaries which apparently have a low permeability to sodium (Hahn & Hevesy, 1940). The retinal circulation is in effect a portion of the cerebral vascular system and a relative impermeability of these capillaries to sodium and potassium would probably account for the relative impermeability of the posterior part of the barrier, separating the plasma and vitreous body, to these ions.

On this basis, then, we may tentatively classify penetrating compounds; a low value of K'_A/K'_V represents an ability to penetrate the vitreous body both from the ciliary body and the more posterior region—thiocyanate, urea and the monosaccharides are examples; a high value, on the other hand, indicates a relative impermeability of the more posterior part of the barrier, so that penetration is predominantly by way of the ciliary body—examples are sodium, potassium, amino-acids, creatinine, and possibly penicillin and glycerol. We must remember, however, that errors involved by the approximation alluded to above will be greater with substances such as sodium and potassium which, on the basis of studies on the frozen vitreous body, may be presumed to enter predominantly by way of the ciliary body. The rates of penetration of these ions may therefore be rather larger than is indicated by the computed values of K'_V. It might therefore be possible to find two substances giving the same value of K'_V one of which penetrated from the ciliary body alone and the

other penetrating over the whole area of contact, the former actually having the larger true permeability.

We may now discuss the role of the iris in the formation and circulation of the aqueous humour. Earlier studies on the sugars (Davson & Duke-Elder, 1948) indicated that the rate of penetration tended to increase with decreasing size of the pupil, i.e. with increasing area of the iris exposed. It was tentatively concluded, on these grounds, that diffusion from the anterior surface of the iris was an important factor in the penetration of sugars into the aqueous humour. Histological evidence, moreover, supports this view since it would appear that the outermost, endothelial, layer of the iris is incomplete, so that the only barrier separating the blood plasma in the vessels of the stroma of the iris from the aqueous humour is the capillary endothelium. These capillaries belong to the uveal, as opposed to the retinal system and it is difficult to believe that they could be impermeable to the various non-colloidal substances considered in this paper. The iris may not, however, be as all-important for diffusion into the aqueous humour as these considerations would seem at first sight to demand; the sum of clinical and physiological evidence indicates the existence of a current through the pupil of aqueous humour which is drained away from the specialized region in the angle of the anterior chamber; the possibility also arises of a bulk reabsorption through the capillaries of the iris. If the capillaries of the iris represented a region favourable for reabsorption this bulk movement of fluid out of the eye would make the penetration of dissolved substances into the eye relatively more difficult than through the capillaries of the ciliary body which seem adapted for ultrafiltration, being very similar to the glomerular tufts of the kidney. We must therefore consider the possibility that, at least for some substances, the main route of penetration into the aqueous humour is by way of the ciliary body associated with flow through the pupil, diffusion from the anterior surface of the iris being a secondary factor. It is worth noting that if this were the exclusive mechanism for the penetration of a substance into the aqueous humour, i.e. if it entered and left the eye by a bulk flow, its rate of penetration would be determined entirely by the rate of drainage and it should be possible to estimate this from the observed value of K'_A. For sodium, with a value of 37·6 this would correspond to a drainage rate of 14·4 mm.^{3}/min., in other words, 14·4 mm.^{3} of an ultrafiltrate of plasma would have to enter the anterior chamber every minute to account for the rate of penetration of radioactive sodium into the aqueous humour.* It should be emphasized that

* This is the essential basis of Kinsey & Grant's (1942) estimate of the rate of drainage in rabbits; they assume that salts are secreted into the eye at a rate proportional to their concentration in the blood and removed by drainage. Formally this would be equivalent to a simple filtration-reabsorption mechanism. It is unlikely that salts enter only by secretion; even if they did however, the drainage rate computed in this way could conceivably be in error owing to diffusion into the vitreous body which, in the rabbit with its shallow anterior chamber, represents a large proportion of the intra-ocular fluids.

if this were indeed the only mechanism by which sodium could enter the anterior chamber this estimate of the rate of drainage would still be seriously in error since the assumption is made that an ultrafiltrate of plasma passes through the pupil into the anterior chamber; our experiments have shown that radioactive sodium diffuses from the region of the ciliary body backwards into the vitreous body; an ultrafiltrate formed in the ciliary region would therefore lose radioactive sodium to the vitreous body and flow through the pupil in a lower concentration than that expected of an ultrafiltrate of plasma.

In conclusion, we may draw attention to a discrepancy between the values for K'_A reported here with the intact cat and those found by Davson & Quilliam (1940) with the isolated head preparation; the values for potassium, sodium and chloride in the latter instance were considerably smaller, and it would appear that the eye in the isolated head is not completely normal; presumably portions of the capillary bed are cut out of circulation by blocking of small arteries, a factor that would reduce the efficiency of the ciliary processes, etc. This supposition is borne out by the findings that in the isolated head sodium and chloride penetrated considerably more slowly than potassium; in the experiments on sodium and chloride it was necessary to make the blood very seriously abnormal—dialysing a part of the sodium or chloride away from it—and it was with some difficulty that the preparations could be maintained 'alive'.

SUMMARY

1. The rates of penetration of sodium, potassium, thiocyanate, phosphate, glycine, alanine, creatinine and urea into the aqueous humour and vitreous body of the cat have been measured and compared with those for monosaccharides and sucrose.

2. For penetration into the aqueous humour the order of decreasing rates is: $CNS^- >$ Na, K $>$ monosaccharides $>$ glycine, alanine, creatinine, urea $>$ sucrose, phosphate; for the vitreous body the order is: $CNS >$ monosaccharides $>$ Na, K, urea $>$ glycine, alanine, creatinine, sucrose, phosphate.

3. The sugars, thiocyanate and urea seem to be differentiated from the remaining substances studied in being able to penetrate the vitreous body readily from the posterior regions of its surface, i.e. from the choroidal and retinal circulations.

4. The results indicate in general that the barriers separating the intra-ocular fluids from the blood plasma are not simple pore structures and emphasize the importance of chemical structure in determining the rate of penetration of a substance.

We acknowledge with appreciation the technical assistance of Miss P. A. Matchett. The whole of the expense of this work was defrayed by the Medical Research Council to which one of us (D.M.M.) is indebted for a personal grant.

REFERENCES

Aldridge, W. N. (1945). *Analyst.* **69**, 262.

Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby Lockwood.

Danielson, I. S. (1933). *J. biol. Chem.* **101**, 501.

Davson, H. & Danielli, J. F. (1943). *The Permeability of Natural Membranes*, Cambridge University Press.

Davson, H. & Duke-Elder, W. S. (1948). *J. Physiol.* **107**, 141.

Davson, H. & Quilliam, J. P. (1940). *J. Physiol.* **98**, 141.

Duke-Elder, W. S. & Davson, H. (1943). *Brit. J. Ophthal.* **27**, 431.

Folin, O. (1905). *Amer. J. Physiol.* **13**, 45.

Folin, O. & Wu, H. (1919). *J. biol. Chem.* **38**, 81.

Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.

Hahn, L. & Hevesy, G. (1940). *Acta physiol. Scand.* **1**, 347.

Kinsey, M. E. & Grant, W. M. (1942). *J. gen. Physiol.* **26**, 131.

Liddell, E. G. T. & Sherrington, C. (1929). *Mammalian Physiology*, 3rd ed. Oxford University Press.

Maurice, D. M. (1948). *J. Physiol.* **107**, 26 *P*.

Palm, E. (1947). *Acta ophthal., Kbh.*, **25** 139.

Rosner, L. & Bellows, J. G. (1939). *Amer J. Physiol.* **125**, 652.

v. Sallmann, L. & Meyer, K. (1944). *Arch. Ophthal., N.Y.*, **31**, 1.

Silberstein, F., Rappaport, F. & Reifer, I. (1937). *Klin. Wschr.* **16**, 1506.

Somogyi, M. (1930). *J. biol. Chem.* **86**, 655.

Weld, C. B., Feindel, W. H. & Davson, H. (1942). *Amer. J. Physiol.* **137**, 421.

J. Physiol. (1949) 108, 218–225 577.174.5:612.12

ACTION OF ADRENALINE-LIKE SUBSTANCES ON THE SERUM POTASSIUM

By JOHN L. D'SILVA

From the Department of Physiology, St Bartholomew's Hospital Medical College, London, E. C. 1

(*Received* 12 *April* 1948)

It has been shown (D'Silva, 1934; Schwarz, 1934–5; Marc nzi & Gerschman, 1937) that the intravenous injection of adrenaline into the cat, rabbit, or dog, results in a transient though very marked and prompt increase in the serum potassium, which thereafter falls within 5–7 min. to its pre-injection level, then drops below this, and finally returns more slowly to it. In man, Keys (1938), Castlèden (1937–8), and Allott & McArdle (1937–8) failed to detect the initial increase in the concentration of potassium after the intramuscular or intravenous injection of adrenaline. Keys, however, gave the intravenous injection during 1·5–3 min., and he and the other workers mentioned confined their analyses to *venous* blood. D'Silva (1939) and Brewer, Larson & Schroeder (1939) pointed out that an increase in the concentration of potassium in the arterial blood might not appear in venous blood because the tissues remove the excess of potassium very rapidly. When the experiment was adapted to take account of this fact, Brewer *et al.* (1939) found that man's response to adrenaline was like that described above for animals.

As the response of the serum potassium to injection of adrenaline was common to all the species investigated, the next step was confined to a single species, the cat. This next step was to study the effect of various adrenaline-like substances on the serum potassium, in order to discover which groupings in the adrenaline molecule are of importance in eliciting the response.

METHODS

Cats anaesthetized with ether, followed by chloralose, were used. The potassium content of the serum from samples of arterial blood was determined with an accuracy of 2–3% by the method of Kramer & Tisdall (1921) as modified by Hubbard (1933). After preparation, the animal was left for 1–2 hr. to settle down and then successive injections of the drug to be tested were given at intervals of not less than ¾ hr.

A control blood sample (about 5 ml.) was withdrawn from the carotid or femoral artery and then the drug was injected, as its hydrochloride or acetate dissolved in 1 ml. of 0·9% sodium chloride. The solution was washed into the vein through the needle with a further 0·5 ml. of saline. In every case, the injection was completed in 10 sec. A second sample was withdrawn during a period of

10 sec., starting 55 sec. after the commencement of the injection. With this standard procedure, the results of different injections in the same cat could be considered comparable; with this speed of procedure, the rapid changes in serum potassium concentration could be adequately followed.

RESULTS

The variation in the response to adrenaline injected into a forelimb vein

In a previous investigation (D'Silva, 1937) it was found that, when 0·1 mg. adrenaline was injected into the blood used to perfuse the liver, 12–15 mg. potassium were liberated. Smaller doses liberated proportionately less. In the course of many previous experiments, the effect on the serum potassium of 0·05 mg. adrenaline injected into a forelimb vein had often been tested, but the

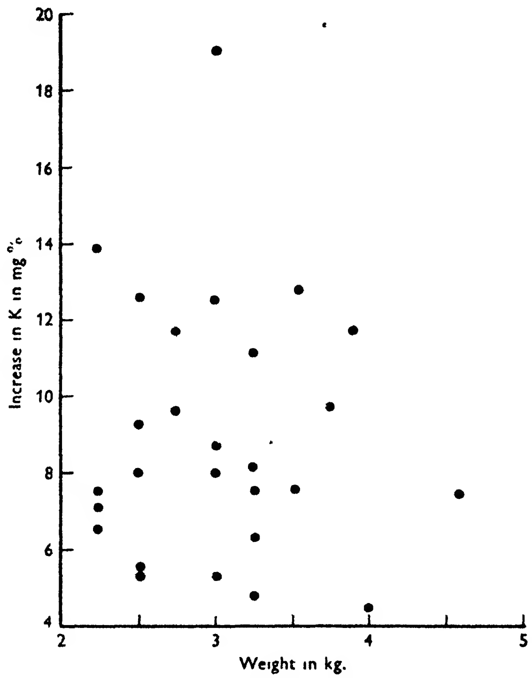


Fig. 1. The relationship between the weight of the animal in kg. and the increase in serum potassium in mg. % after adrenaline.

results showed that the increase in serum potassium one minute after the injection was not constant from animal to animal. One possible reason for the inconstancy might have been the varying weights of the animals. Since the dose of adrenaline was constant, the concentration of adrenaline in the blood reaching the liver would be lower in the larger animals, and so the concentration of potassium in the blood leaving the liver would, no doubt, also be lower. The increase in potassium concentration in the arterial blood would thus be less in a large than in a small animal. In Fig. 1 the rise in serum potassium is

plotted against the weight of the animal. There appears to be no correlation between the weight of the animal and its response to adrenaline. The variation in weight can account for only a small part of the variation in the rise of serum potassium.

It was previously (D'Silva, 1937) shown that suitably spaced successive injections of adrenaline into an animal produced approximately equal increases in the concentration of potassium in the serum.

The action of adrenaline-like substances on the serum potassium.

The effect on the serum potassium of an adrenaline-like substance was always compared with that of adrenaline in the same animal. A preliminary experiment with graded doses of the drug, gave an approximate figure for its activity relative to that of adrenaline. An example is given in Protocol 1, from an experiment in which the action of epinine was investigated. This experiment shows that 0·017 mg. L-adrenaline/kg. is equivalent in its effect on the serum

Protocol 1

Cat. ♀. 3·25 kg. Ether-chloralose. Waited 1 hr. after chloralose.
E = epinine; A = L-adrenaline.

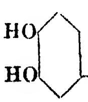
Intravenous dose (mg.)	Serum K (mg. %) Before	After 1 min.	Increase in K (mg. %)
0·5 E	13·1	17·3	4·2
1·0 E	12·2	23·6	11·4
2·0 E	14·5	29·5	15·0
0·05 A	15·3	21·8	6·5
0·05 A	15·3	21·6	6·3
0·05 A	15·0	22·6	7·6

0·05 mg. L-adrenaline is equivalent to 0·5–1·0 mg. epinine.

Cat. ♀. 3 kg. Ether-chloralose. Waited 1 hr. after chloralose.

Intravenous dose (mg.)	Serum K (mg. %) Before	After 1 min.	Increase in K (mg. %)
0·5 E	13·5	16·4	2·9
0·05 A	12·5	20·6	8·1
1·0 E	12·6	23·2	10·6
0·05 A	12·7	23·2	10·5
1·0 E	14·4	24·7	10·3

TABLE 1. The relative effect on the blood potassium of substances related to adrenaline

Substance tested	 —CH	—CH–	NH	Dose (mg./kg.)	Increase in serum K (mg %)	Relative activity
L-Adrenaline	OH	H	CH_3	0·017	8·9	100
D-Adrenaline	OH	H	CH_3	0·074	6·2	17
DL-Adrenaline	OH	H	CH_3	0·03	7·5	40
DL-Arterenol	OH	H	H	0·052	11·7	25
DL-Corbasil	OH	CH_3	H	0·24	9·3	4
DL-Colephrine	OH	CH_3	CH_3	0·23	10·5	7
Epinine	H	H	CH_3	0·29	6·4	5

potassium to 0·33 mg. epinine/kg. The ratio of the doses is 1:20, but the result can only be an approximation.

The substances listed in Table 1 rapidly (within 1 min.) increased the concentration of potassium in the serum. Column 6 shows the average rise in serum potassium for the dose stated in column 5. None of the substances tested was as active as L-adrenaline, which was assigned, arbitrarily, the figure 100 for its activity. The relative activities of the compounds listed in column 7 were obtained by the method described above for epinine.

The following deductions can be made from Table 1. (*a*) The reaction exhibited a considerable degree of specificity, for small modifications in the structure of the adrenaline side-chain considerably altered the activity of the drug. (*b*) Removal of the centre of asymmetry, as in epinine, considerably diminished the potassium-releasing action of the drug. (*c*) A change in the optical configuration of the molecule was important; e.g. D-adrenaline had 17% of the activity of the L-isomeride. (*d*) The introduction of a methyl group into the α-position considerably diminished the potassium-mobilizing action of the drug, as shown by the relative effect of DL-colephrine and DL-adrenaline. (*e*) The change of the terminal $NHCH_3$ group in DL-adrenaline into NH_2 in arterenol was associated with diminished activity; DL-arterenol had about 60% of DL-adrenaline's effect on the serum potassium. (*f*) All the compounds listed in Table 1 are derivatives of 3:4-dihydroxyphenylethylamine. All possess the catechol nucleus and have either one methyl group, or none, attached to the nitrogen of the side-chain.

Duration of action and 'after fall'. After removal of a control blood sample, the drug was injected and the serum potassium concentration was followed. In every case after the injection of equivalent doses of the different drugs listed in Table 1 (0·05 mg. L-adrenaline was the reference dose), the concentration of potassium returned to normal within 6 min. Thereafter, the serum potassium fell below the initial level in every case and returned to its original value more slowly. In both these respects the drugs tested reacted in the same way as L-adrenaline.

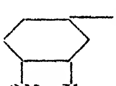
None of the drugs listed in Table 1 when injected intravenously increased the serum potassium 1 min. after injection when the portal area was excluded from the circulation (cf. D'Silva, 1936).

The effect of some other substances on the serum potassium

In Table 2 the results of tests with five other compounds are summarized. It will be seen that the first four of these have side-chains identical with those of compounds listed in Table 1, but that they have no effect on the serum potassium. They differ from the compounds in Table 1 only in the structure of the nucleus. The last compound in the list, β-hydroxy-β-(3:4-dihydroxyphenyl)-ethyldimethylamine ('Methadren'), is very similar in chemical struc-

ture to adrenaline. It differs from it only in having a second methyl group attached to the nitrogen, and is therefore a tertiary amine. This difference in structure also results in the suppression of potassium-mobilizing activity.

Table 2. The effect on the blood potassium of substances related to adrenaline

Substance tested			CH—	CH—	NH	Dose (mg./kg.)	Increase in K (mg. %)
Tyramine	OH	H	H	H	H	2·3	0·2
						1·9	0·7
Ephedrine	H	H	OH	CH_3	CH_3	2·1	0·6
						4·3	1·0
β-Hydroxy-β-phenylethylamine	H	H	OH	H	H	1·7	1·0
						3·3	1·5
DL-*p*-Sympatol	OH	H	OH	H	CH_3	3·3	2·3
						25·0	1·9
'Methadren'	OH	OH	OH	H	*$(CH_3)_2$	0·02	1·2
						0·04	0·0
						1·8	1·2
						3·70	0·8

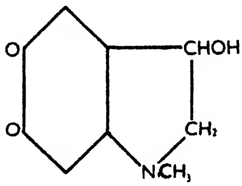
* Terminal group is $\cdot N(CH_3)_2$.

It seems, therefore, that both a catechol nucleus and a hydrogen atom attached directly to nitrogen in the adrenaline side-chain are necessary to endow the compound with potassium-mobilizing activity.

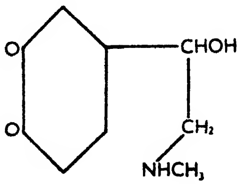
The action of adrenochrome on the serum potassium. The method of preparation, communicated to me by Mr Harley Mason, consisted in shaking a solution of L-adrenaline in 2% formic acid in methyl alcohol with a suspension of silver oxide for 1 min. at 30–40°. The solution was filtered, the residue washed with a little methyl alcohol, and the filtrate cooled to −20° C. Adrenochrome (I) (cf. Green & Richter, 1937) separated as a dark red product which decomposed at 130°. The major portion of the product crystallized in prisms, but in the three preparations which were made, a small proportion of dark brown amorphous material (? melanin) could be seen with the aid of a microscope. This may have been due to the difficulties of manipulation because of the small scale of the preparation (0·3 g. L-adrenaline was converted into adrenochrome), or to the fact that none of the three samples was recrystallized. The material dissolved in 0·9% NaCl to give a dark red solution and even a dilution of 1:10,000 was strongly coloured. When 0·5 ml. of this solution (i.e. the equivalent of about 0·05 mg. adrenaline) was injected into a cat under chloralose, there was no prompt increase in the concentration of potassium in the serum.

In one trial, the oxidation of adrenaline was carried out but adrenochrome was not isolated in crystalline form. Instead, the dark red reaction mixture was diluted with 0·9% saline to give a concentration of adrenochrome of 1:10,000, assuming that the adrenaline had been converted quantitatively into adrenochrome. The solution was filtered. 0·5 ml. of the solution when injected

into a cat produced no significant change in the serum potassium within a minute. The red solution was tested for adrenaline activity on the cat's nictitating membrane and was found to contain not more than 3 μg. in 0·5 ml. of solution, i.e. about 6% of the original adrenaline activity remained. This was too small an amount of adrenaline to have a detectable effect on the serum potassium by the intravenous route used.



(I)



(II)

The effect of alkali-treated adrenaline (*A.T.A.*). Shaw (1941) described the effect of 1·5N-Na_2CO_3 on weak solutions of adrenaline. When a 1:10,000 solution of L-adrenaline (2 ml.) was treated with 1·5N-Na_2CO_3 (0·2 ml.) for 3 min., a compound was formed with the physiological actions of adrenaline on the heart and intestine, but which differed from adrenaline in its chemical properties. This led him to ascribe the formula (II) to the product which, however, he was unable to isolate because of its instability. Shaw's experiments have been repeated and confirmed.

When 0·5 ml. of a solution prepared as described above was injected into a forelimb vein, there was a prompt increase in the concentration of potassium in the serum. A.T.A. was found to be 60–70% as active as L-adrenaline in raising the serum potassium, but it must be noted that treatment with alkali, besides yielding the quinone (II), also gives an indole (? adrenochrome) and another inactive substance as by-products (Shaw, 1941). These side reactions might easily account for the diminution in potassium-releasing activity.

The duration of action of A.T.A. is like that of L-adrenaline, and it produces a similar 'after-fall'. When the portal area was excluded from the circulation, the intravenous injection of A.T.A. did not increase the concentration of potassium in the serum. In its effects on the serum potassium, therefore, A.T.A. closely resembles the action of L-adrenaline.

DISCUSSION

The observation that all the substances listed in Table 1 were capable of producing prompt and large increases in serum potassium after intravenous injection suggested that this reaction might occur as a result of their preliminary conversion in the body into the corresponding adrenochromes; for adrenochromes are formed only by compounds which, like these, are derived from 3:4-dihydroxyphenylethylamine. Because, however, adrenochrome itself,

when injected, did not raise the serum potassium, it is concluded that the preliminary formation of adrenochrome is not involved in the potassium-liberating reaction.

Three other known mechanisms by which adrenaline can be inactivated might play a part in increasing the serum potassium.

(*a*) *Auto-oxidation.* This is probably insignificant in vivo because adrenaline is very rapidly removed from the blood stream, and the tissues protect the drug from oxidation (Wiltshire, 1931).

(*b*) *Conjugation.* Richter & MacIntosh (1941–2) have shown that L-adrenaline, after injection, is excreted in the urine of a rabbit in a form in which the phenolic hydroxyl groups are masked. After hydrolysis of the urine with acid, a substance which gives the chemical and physiological tests for adrenaline is formed. DL-Corbasil, epinine, D- and L-adrenaline are similarly excreted *slowly* by man in a conjugated ('detoxified') form (Richter, 1940). On the other hand, the changes which occur in the serum potassium are very rapid. It is unlikely, therefore, that the conjugated compound is directly responsible for the mobilization of potassium which occurs after the intravenous injection of adrenaline.

(*c*) *Adrenaline oxidase.* This enzyme is found in liver and has been shown, in vitro (Blaschko, Richter & Schlossmann, 1937) to oxidize adrenaline and some similar compounds, the grouping $:C.CH_2.N:$ in the side-chain being necessary for the reaction. This reaction does not account for the effect of the drugs tested, because DL-corbasil is unaffected by adrenaline oxidase and yet it increases the concentration of potassium in the serum. On the other hand, tyramine is acted on by the enzyme and has no potassium-releasing activity.

There is no evidence, therefore, that any known oxidation product of adrenaline takes part in the reaction except the, as yet, unisolated open-chain quinone (II), which is probably produced when adrenaline is treated with alkali. It appears likely that, with each of the active compounds, the potassium-releasing effect is to be ascribed to the unmodified molecule, rather than to a detoxication product.

SUMMARY

1. D-, L- and DL-Adrenaline, DL- arterenol, epinine, DL-corbasil and DL-colephrine when injected intravenously into cats produce prompt increases in the concentration of potassium in the serum. The sequence of changes in the serum potassium is the same with each drug. None increases the serum potassium when the portal area is excluded from the circulation.

2. Tyramine, ephedrine. β-hydroxy-β-phenylethylamine, DL-P- sympatol, and β-hydroxy-β-(3:4-dihydroxyphenyl)ethyldimethylamine ('Methadren') after injection do not increase the concentration of potassium in the serum.

3. The reaction appears to depend on the catechol nucleus and on the presence of a hydrogen atom linked directly with nitrogen in the side-chain.

4. No known oxidation product of adrenaline gives this reaction, but it seems probable that the, as yet, unisolated 'open-chain quinone' does.

Most of these experiments were carried out before the recent War. I am indebted to Messrs I. G. Farbenindustrie, Hoffmann-La Roche and Co., and C. H. Boehringer Sohn, for some of the compounds which were tested.

REFERENCES

Allott, E. N. & McArdle, B. (1937–8). *Clin. Sci.* **3**, 229.
Blaschko, H., Richter, D. & Schlossmann, H. (1937). *J. Physiol.* **89**, 39*P*.
Brewer, G., Larson, P. S. & Schroeder, A. R. (1939). *Amer. J. Physiol.* **126**, 708.
Castleden, L. I. M. (1937–8). *Clin. Sci.* **3**, 241.
D'Silva, J. L. (1934). *J. Physiol.* **82**, 393.
D'Silva, J. L. (1936). *J. Physiol.* **86**, 219.
D'Silva, J. L. (1937). *J. Physiol.* **90**, 303.
D'Silva, J. L. (1939). *St Bart's Hosp. Rep.* **72**, 303.
Green, D. E. & Richter, D. (1937). *Biochem. J.* **31**, 596
Hubbard, R. S. (1933). *Amer. J. Physiol.* **100**, 558.
Keys, A. (1938). *Amer. J. Physiol.* **121**, 325.
Kramer, B. & Tisdall, F. F. (1921). *J. biol. Chem.* **48**, 223.
Marenzi, A. D. & Gerschman, R. (1937). *C.R. Soc. Biol., Paris*, **124**, 382.
Richter, D. (1940). *J. Physiol.* **98**, 361.
Richter, D. & MacIntosh, F. C. (1941–2). *Amer. J. Physiol.* **135**, 1.
Schwarz, H. (1934–5). *Arch. exp. Path. Pharmak.* **177**, 628.
Shaw, F. H. (1941). *Aust. J. exp. biol.* **19**, 151.
Wiltshire, M. O. P. (1931). *J. Physiol.* **72**, 88.

J. Physiol. (1949) 108, 226–232 612.741:595.7

THE EXCITATION AND CONTRACTION OF THE FLIGHT MUSCLES OF INSECTS

By J. W. S. PRINGLE

From the Department of Zoology, University of Cambridge

(*Received* 3 *May* 1948)

The beating of the wings of all the higher orders of insects except the dragonflies (Odonata) is produced by indirect muscles which are attached not directly to the bases of the wings, but longitudinally and vertically across the box-like thoracic cavity (Text-fig. 1). By the alternate contraction of these two sets of muscles the thorax is distorted in such a way that the wings are moved on their basal articulation. In addition to the indirect musculature, which in the Diptera and Hymenoptera occupies the greater part of the volume of the thorax, several smaller muscles are attached directly to the wing bases; these alter the position and incidence of the wings and are usually considered to be the means of control of flight, the indirect muscles providing the energy for the wing beats.

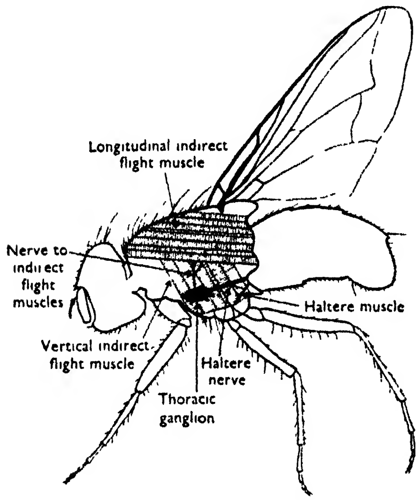
It has been known for some time (Keilich, 1918) that the indirect muscles differ considerably in their histological appearance from other insect muscles. The large, striated muscle fibres, which stretch from one side of the thorax to the other, are more or less completely divided longitudinally into well-defined myofibrils, which may be only loosely bound together into fibre units. In the muscid Diptera a sarcolemma and some sarcoplasm are present, but the fibrils may be teased out even in the unfixed tissue, and are readily seen after fixation in hot alcohol, which appears to dissolve away the boundary membrane of the fibre.

There has been no modern physiological investigation of these indirect muscles. This paper describes some observations on the blue-bottle, *Calliphora erythrocephala* Mg., which suggest that they have a number of peculiar properties.

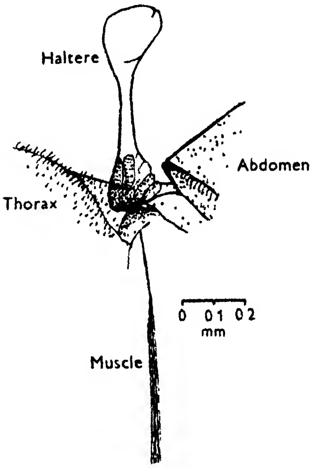
The frequency of the wing beat. Recent observations (Sotavalta, 1947) show that in certain small midges (*Forcipomyia*) the frequency of wing beat is above 1000 beats/sec. Frequencies in the region of 150–300 beats/sec. are commonly found in the larger Diptera (*Calliphora, Musca, Eristalis*). Even the lower

figures represent a rate of muscular action far higher than is found elsewhere in the animal kingdom; a frequency of 1000 beats/sec. involves a complete cycle of contraction and relaxation in 1 msec.

Sotavalta, after a series of experiments on the effect on the frequency of wing beat of altering the air pressure and the loading on the wings, concludes that the inertia of the wing is the main factor controlling the beat frequency. Sellke (1936) and Pringle (1948) describe experiments on the halteres of Diptera (Text-fig. 2) which lead to the same conclusion in the case of these organs, which are modified hind wings. These observations pose an interesting question.



Text-fig. 1.



Text-fig. 2.

Text-fig. 1. Anatomy and innervation of the indirect flight and haltere muscles of *Calliphora*: diagrammatic view of a fly bisected by a vertical median cut.

Text-fig. 2. Ventral view of the left haltere of *Calliphora*. The organ oscillates during flight through an angle of 150° in a vertical plane at a frequency of 100–150/sec.

If the contraction of the muscles is produced, as in all known somatic striated muscles, by the arrival of a nerve impulse at the neuromuscular junction, the frequency must be determined centrally in the thoracic ganglion, and the change in frequency as the loading is altered must be a reflex from sense organs in the wings or in the wing muscles. No sensory endings can be observed histologically in the wing or haltere muscles, and it is known (Pringle, 1938) that the main proprioceptive sense organs in the legs of insects are the campaniform sensilla embedded in the chitinous skeleton. Campaniform sensilla are always found in considerable numbers at the base of the wings and halteres (Vogel, 1911; Pflugstaedt, 1912), and in the halteres they have been shown to be sensitive to the strains produced by the oscillation of the organ (Pringle,

1948). It is highly probable that they are the proprioceptors for the wings. The following experiments, however, suggest that the dependance of wing and haltere frequencies on loading is not a reflex phenomenon.

EXPERIMENTAL

Oscillation of the isolated haltere. The halteres of the higher Diptera are moved by means of a single muscle attached to the cuticle near the base of the organ (Text-figs. 1, 2). Contraction of this muscle moves the haltere upwards, and it returns to a ventral position, when the muscle relaxes, by virtue of the elasticity of the hinge. The other end of the muscle is inserted on the pleuron of the metathoracic segment, so that by dissection outwards in a fly bisected by a vertical median cut it may be exposed without distortion of the skeleton and consequent alteration of its natural length.

Some preparations made in this way show an active oscillation of the haltere, even when the thoracic ganglion is completely removed and all nerves to the muscle are cut. When this occurs, the oscillation may be stopped by touching the haltere with the point of a needle and gently releasing it. If it is now given a slight flick with the needle, the oscillation restarts and builds up to full amplitude in the course of a few cycles. This starting and stopping may be repeated many times. Since the whole of the organ is isolated from the central nervous system, no reflex mechanism can in this case be responsible for the oscillation.

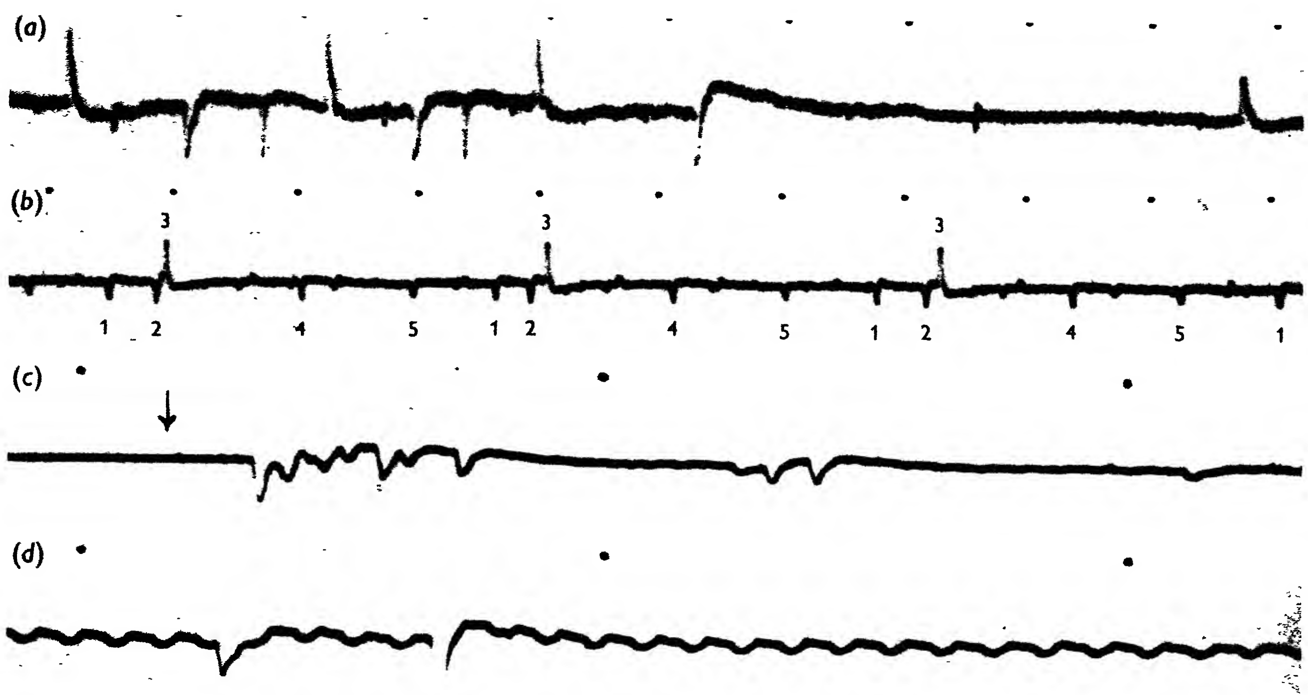
'Anaesthetic flight.' Flies recovering from ether anaesthesia pass through a stage when they fly steadily for several minutes before any obvious reflexes have returned. For example, in an unanaesthetized fly flight is regularly inhibited by contact of the legs with a solid object; this reflex is absent in the 'anaesthetic flight' condition. The dependance of wing-beat frequency on loading is present in this 'anaesthetic flight' as in the normal fly.

Elimination of proprioceptive sense organs. The sense organs on the wings may all be removed by section of the wings at the extreme base. A fly so treated will still make the muscular movements of flight during recovery from ether anaesthesia, the distortion of the thorax being sufficient to produce an audible buzz, in this case at a high frequency, since the inertia of the wings has been reduced almost to zero. If the distortion of the thorax is resisted by pressure dorso-ventrally between two solid objects, the frequency of the buzz is reduced in proportion to the pressure applied. In this case not only are normal reflexes absent due to the anaesthetic, but the probable proprioceptors of the wings have been completely removed.

The conclusion from these experiments is that the frequency of contraction of the muscles is not determined centrally in the ganglion, but is controlled directly by the loading upon them.

Photomicrograph of the branching of a single nerve fibre on the surface of a portion of the vertical indirect flight muscle of *Calliphora*: from a fresh preparation injected with Rongalit methylene blue.

To face p. 228



Electrical records from the longitudinal indirect flight musculature of *Calliphora*. Time marks $\frac{1}{10}$ sec. (*a*) During a short period of wing movements after full recovery from anaesthesia. (*b*) During the steady 'anaesthetic flight': the distinctive spikes are numbered. (*c*) At the start of wing movements, the arrow marks the approximate instant of removal of contact stimuli to the legs. (*d*) At the end of a period of flying: higher amplification.

Effects of electrical stimulation

Wing muscles. If a fly is bisected vertically with a sharp razor blade, no gross damage is done to the musculature of the thorax, and the two halves may be used for physiological experiments. That there is little upset to the nervous system is shown by the fact that the half containing the thoracic ganglion shows the normal leg reflexes (Pringle, 1940).

In such a preparation the indirect flight musculature cannot be made to contract by electrical stimulation. Direct current or condenser discharges (CR $\simeq 2 \times 10^{-4}$ sec.) applied through platinum wire electrodes placed in a variety of positions on the longitudinal muscles fail to elicit any movement visible under a high-power dissecting microscope, at any frequency of stimulation from 1 to 400 shocks/sec., and up to intensities sufficient to produce electrolysis.

Haltere muscle. Similar experiments have been made with the haltere muscle. In this case the result is usually the same, but in one or two preparations a slight movement of the haltere has been observed at low frequencies of stimulation, the individual twitches fusing into a smooth tetanus at about 10 shocks/sec. The small size of the haltere (0·7 mm. long) has precluded recording of the contractions, and the results have been observed visually. The movement produced in this way is slow and quite different from the rapid flick characteristic of the normal oscillation.

If condenser discharge shocks are applied to a haltere muscle showing its normal rhythmic activity, the contractions bear no relation to the shocks at any frequency of stimulation between 40 and 400/sec. The movement of the haltere has been observed stroboscopically during stimulation, and the frequency of oscillation is unaffected by the electrical stimuli.

Innervation

The nerve supply to the indirect flight muscles of *Calliphora* is readily seen in a specimen injected 1–2 hr. previously with a small quantity of Rongalit methylene blue (prepared as described by Pantin, 1946). Each portion of the musculature is supplied by a single nerve fibre (Pl. 1), which branches freely over the surface of the muscle. There is no sign of the double or triple innervation characteristic of the leg musculature of Crustacea (Wiersma, 1941) and insects (Pringle, 1939, and unpublished histological data). The nerve fibres supplying both the longitudinal and vertical indirect flight muscles leave the ganglion in a single common trunk (Text-fig. 1) which passes between the two sets of muscles and sends branches into each of them. There are not more than five or six nerve fibres to the whole of the indirect musculature of one side.

Electrical response

Electrical records from the flight muscles of *Calliphora* have been obtained with a concentric electrode made from a no. 19 hypodermic needle and a piece of enamelled copper wire. The electrodes were connected through a 0·05 μF. condenser (to avoid movement artefacts due to grid current) to a condenser-coupled amplifier and oscilloscope.

Pl. 2 shows the type of record obtained with the electrode inserted from the top of the thorax into the longitudinal indirect flight muscle of one side. The intact fly is supported on a piece of wire waxed to the centre of the top of the thorax (technique of Hollick, 1940), and has a stream of air directed at it from in front. Movements of the wings are readily elicited in a fly mounted in this way by removal of a piece of paper from the grasp of the legs.

At each cycle of wing beat there is a small electrical effect, 200–500 μV. in amplitude (Pl. 2*a*, *d*). When the electrode is placed accurately in the centre of the longitudinal muscle the deflexion of the trace occurs once per cycle (Pl. 2*d*); if it is between the two muscle sets the combined effect produces a nearly sinusoidal trace (Pl. 2*a*). This regular effect is accompanied by individual "spikes" of much larger amplitude (approx. 5–10 mV.), which bear no relation to the cycles of wing beat. At the beginning of the flight (i.e. immediately the contact stimulus to the legs is removed) there is a rapid burst of these large spikes (Pl. 2*c*), and their frequency then falls to a level which is maintained during the wing movements. During 'anaesthetic flight' there is a continuous discharge (Pl. 2*b*).

The records show that at least five different types of spike are distinguishable by their polarity, amplitude and rhythm (Pl. 2*b*). The form of the electrical record is typical of insect muscle action potentials (cp. the leg muscle records in Pringle, 1939). The different spikes are probably due to different portions of the musculature being innervated by different single fibres; the constancy of the rhythm in each unit is remarkable (Pl. 2*b*). At the end of the flight activity the electrical effect at wing-beat frequency dies away in a gradual manner over several cycles (Pl. 2*d*). The last large spike occurs, in this experiment, $\frac{1}{5}$ sec. before the wing beats cease.

DISCUSSION

It is clearly impossible to explain the above results in terms of a neuromuscular system similar to anything so far described. The most probable interpretation is as follows:

(*a*) The arrival of an impulse in the nerve fibre supplying a portion of the indirect musculature sets up an excitation process in the muscle fibres which produces an electrical spike. Whether this is a true propagated action potential or an exaggerated end-plate potential remains undetermined (cp. Katz & Kuffler (1946) on the Crustacean leg neuromuscular junction).

(*b*) Excitation of the muscle fibres alters the state of the contractile elements in such a way that they become sensitive to the stimulus of stretching. On being stretched, the myofibrils respond with a twitch-like contraction whose duration is controlled by the loading, and then relax.

(*c*) Contraction of one set of muscles stretches the antagonist, which in turn responds with a twitch-like contraction. In the special case of the haltere, the contraction of the single muscle sets in motion a mechanically resonant system and the muscle is re-excited by the elastic rebound.

(*d*) This myogenic rhythm continues for so long as the excitation of the muscle fibres is maintained by the arrival of nerve impulses above a critical frequency. When the nerve impulses stop the muscular contractions die out owing to the failure of one or other set of muscles to supply enough energy to maintain the rhythm.

The independence of the excitation process (as measured by its electrical spike) and the contraction process in the myofibrils is the most interesting feature of these experiments, and is in contrast to the properties of the other type of insect muscle studied by Pringle (1939) in the legs of the cockroach and by Heidermanns (1931) in the flight muscles of dragonflies. In both those preparations a single electrical stimulus to the muscle fibres was accompanied by a single twitch, as in vertebrate striated muscle.

The extreme sensitivity of the contraction process to the mechanical conditions also represents a specialization to the peculiar function of these indirect flight muscles. To produce an active contraction of the myofibrils it is evidently necessary both that the system should be in an excited state and that the mechanical conditions should be correct. When the thorax is vertically bisected, the loading on the muscles is altered by an upset of the antagonism between the longitudinal and vertical bundles and, although the muscles may be excited by electrical stimulation, no contraction occurs.

The fact that a high frequency of wing beat can be produced by this system implies that the latency between the stimulus of stretching and the response of the myofibrils is very short. This may point to the processes linking the surface excitation phenomena with the contractile process as the main cause of the latent period of normal muscles.

The small electrical effect of the actual contraction is significant for the physiology of the fly as a whole. If conducted action potentials were produced in the thoracic musculature at each cycle of wing beat, it is difficult to see how the large currents generated could fail to spread to the other muscles and to the thoracic ganglion, all of which lie in the restricted space of the insect thorax. At the frequency at which the spikes actually occur (about 3 per sec. in each unit in Pl. 2*b*) the direct flight and the other tonically contracting muscles of the thorax will be almost unaffected.

SUMMARY

1. The effect of loading on the frequency of the wing beat and haltere oscillation of Diptera is a property of the indirect flight muscles, and is not a reflex phenomenon.

2. The indirect flight muscles of *Calliphora* cannot be made to contract by electrical stimulation after exposure by vertical bisection of the thorax.

3. Electrical records from the indirect flight muscles show a small effect at wing-beat frequency accompanied by spikes of large amplitude.

4. The results suggest that an impulse in a nerve fibre supplying the indirect flight muscles sets up an electrical spike in the muscle fibres, and as a result of this the myofibrils are brought into a state of excitation in which they contract actively when stretched.

REFERENCES

Heidermanns, C. (1931). *Zool. Jb., allg. Zool. Physiol.*, **50**, 1.
Hollick, F. S. J. (1940). *Philos. Trans.* **230**, 357.
Katz, B. & Kuffler, S. W. (1946). *Proc. Roy. Soc.* B, **133**, 374.
Keilich, J. (1918). *Zool. Jb., Anat. u. Ontog.*, **40**, 515
Pantin, C. F. A. (1946). *Notes on Microscopical Methods for Zoologists.* Cambridge University Press.
Pflugstaedt, H. (1912). *Z. wiss. Zool.* **100**. 1.
Pringle, J. W. S. (1938). *J. exp. Biol.* **15**, 114.
Pringle, J. W. S. (1939). *J. exp. Biol.* **16**, 220.
Pringle, J. W. S. (1940). *J. exp. Biol.* **17**, 8.
Pringle, J. W. S. (1948). *Philos. Trans.* **233**, 347.
Sellke, K. (1936). *Z. wiss. Zool.* **148**, 465.
Sotavalta, O. (1947). *Acta ent. fenn.* **4**. Helsinki.
Vogel, R. (1911). *Z. wiss. Zool.* **98**, 68.
Wiersma, C. A. G. (1941). *Biol. Symp.* **3**, 259.

J. Physiol. (1949) 108, 233–245 612.79.015:547.781.5

THE EFFECT OF PROTEIN HYDROLYSATES (LEUKOTAXINE) ON SKIN-HISTAMINE IN CATS

By J. DEKANSKI

From the Department of Pharmacology and Polish Medical School, University of Edinburgh

(*Received* 14 *May* 1948)

In 1936 Menkin isolated from human exudate a crystalline substance which possessed chemotactic properties (Menkin, 1937) in addition to the ability to increase capillary permeability on intradermal injection. This substance, which Menkin called 'leukotaxine' was found to display none of the properties of histamine (Menkin, 1936, 1938, 1939) and to bear no relationship to acetylcholine, adenylic compounds or the 'spreading factor' (Menkin, 1937; Menkin & Kadish, 1938). Its physical and chemical properties were consistent with its being a polypeptide of low molecular weight. Additional evidence has been obtained by treating inactive blood serum or crystalline albumin with trypsin. The digest contains both the permeability and the chemotactic factor (Menkin 1938, 1940). Duthie & Chain (1939) repeated these investigations using 'leukotaxine' obtained by the enzymic hydrolysis of a number of proteins and confirmed the physiological properties of leukotaxine. They believed these effects to be due, not to a single substance but to several polypeptides.

While the chemotactic ability of leukotaxine *per se* has not been disputed, considerable controversy has arisen with regard to its property of increasing capillary permeability. It has been suggested (Bier & Rocha e Silva, 1939) that the active permeability factor of exudate is, in fact, histamine and not leukotaxine; or alternatively, that leukotaxine from exudates and leukotaxine from the enzymic hydrolysates of protein induce increased capillary permeability through the mediation of histamine (Rocha e Silva & Dragstedt, 1941). These views have been contested by Menkin (1940) and Cullumbine & Rydon (1946), and Cullumbine (1947). Duthie & Chain (1939) concluded from their investigations that whatever may be the role of histamine in the initial stages of inflammation or in mild temporary skin stimulation, these polypeptides must be largely responsible for the permanent increase in capillary permeability which always occurs in the later stages of inflammation after proteolysis in the body has begun.

In an effort to throw further light on this problem it was decided to determine the amounts of extractable skin-histamine at various intervals after treatment with leukotaxine, histamine and moderate heat and then to compare the results obtained in normal animals, with those obtained after the injection of trypan blue.

METHODS

In the following 'fibrin leukotaxine' and 'exudate leukotaxine' are used for convenience and do not represent any chemically identified compound. It is also too early to say that all properties of leukotaxine obtained from the peptic blood fibrin digest used in these experiments are identical with those of leukotaxine extracted from human pus.

Cats were used in the experiments described here because the amount of extractable histamine of the normal skin of these animals was found quite constant. The cat's skin is much more sensitive to intradermal injections of histamine than rabbit's skin. The local anti-histamine effect of 'neoantergan' was also more marked in the cat's skin than in the rabbit's skin.

The cats were prepared on the previous day by shaving each flank. In the first series of experiments (1 and 5 hr. duration) they were anaesthetized with sodium pentobarbitone ('Veterinary Nembutal', Abbott Laboratories, Ltd.), given intravenously after induction with ether. Four areas were then outlined on each flank by means of a rubber stamp, so as to obtain eight patches of skin of equal surface area. In each experiment, one such outlined area was burnt at 60° C. for 60 sec. using the technique previously described (Dekanski, 1947). Another of the delineated areas of skin served as a control. This patch remained untreated or was injected intradermally with saline, or with neoantergan, or with leukotaxine which had been boiled for 90 min. with conc. HCl. Into the remaining outlined areas of skin were injected either histamine or one of a number of preparations of leukotaxine. In all, three preparations were examined. Two of these were obtained from peptic digests of blood fibrin separated into two fractions by treatment with alcohol. The third leukotaxine preparation was obtained from human pus, (perinephric abscess). In some experiments neoantergan was injected intradermally 15 min. before the histamine or leukotaxine. The volume of the solution injected was in all cases 0·25 c.c. containing 2500–7500 μg. leukotaxine, or 2·5 μg. histamine or 25 μg. neoantergan, the solvent in each case being 0·9 % saline. Immediately the treatment of the various skin areas had been completed, 7–8 c.c. of 1 % trypan blue, purified for physiological purposes (The British Drug Houses, Ltd.) in saline, after being warmed and filtered, was injected into the femoral vein of the cat and the skin observed.

In the second series of more prolonged experiments (12 and 24 hr. duration) the cats were anaesthetized with ether only. Three areas were then outlined on each flank. Each of the outlined areas, except one control normal and occasionally one which was burnt at 60° C. for 60 sec., was then treated with various leukotaxine fractions only. Immediately after burning and intradermal injections of leukotaxine, 1 % trypan blue in saline was injected into the radial vein. After 30 min. the animals were carefully dressed, placed in the cages in a warm room and allowed to recover.

In all these experiments at various intervals after treatment, the outlined patches of the skin were removed, weighed and extracted for the estimation of histamine.

Preparation of leukotaxine. A suspension of 10 g. blood fibrin (The British Drug Houses, Ltd.) in 200 c.c. H_2O was left overnight, and the following morning conc. HCl added, drop by drop, to the suspension until it became thick. 1 g. of a preparation of pepsin dissolved in 5 c.c N/100 HCl was then added and the pH of the mixture adjusted to 1·8–2·0 (glass electrode). After incubation of the mixture at 37° C. for 1 hr. the digest was cooled to room temperature and the pH adjusted to 8·0 by the addition of 2N-NaOH. To the measured volume of stirred digest solution was added and equal volume of pyridine and two volumes of acetone. After centrifuging off the precipitate, the supernatant was evaporated to dryness under reduced pressure, small quantities of ethanol being added from time to time to aid the evaporation and prevent frothing. The fawn-coloured product 'crude leukotaxine' weighing approximately 6 g. was dried for at least 24 hr. in a vacuum desiccator

in the presence of P_2O_5 and H_2SO_4 to remove the last traces of the solvents. Further purification was carried out by exhaustively extracting the 'crude leukotaxine' with 100 c.c. volumes of ethanol at the boiling-point. After 2 hr. the boiling extract was filtered off on a Buchner funnel and the process continued with a further quantity of solvent. After eight to ten such extractions the ethanol was removed from the combined filtrates by evaporation under reduced pressure. Drying in a vacuum desiccator, for at least 24 hr., yielded a yellow product, the 'alcohol-soluble fraction'. The 'alcohol-insoluble fraction', a pale fawn-coloured amorphous solid, was likewise dried to remove the last traces of ethanol before use. In most cases the solids present in 'crude leukotaxine' were found to distribute themselves almost equally between the two fractions.

The human pus was extracted likewise with pyridine and acetone, the rest of the procedure being as described for the preparation of 'crude leukotaxine'. This 'exudate leukotaxine', weighing approximately 2 g. was an amorphous solid, but green coloured, probably owing to the presence of haemoglobin in the pus.

Extraction, estimation and identification of histamine. The extraction was carried out by the method of Barsoum & Gaddum (1935) as modified by Code (1937). The finely cut skin was ground in a mortar, and extracted for 1 hr. with 10% trichloroacetic acid, using 15 c.c. of acid to 1–2 g. of tissue. After filtering by gravity, an aliquot of 10 c.c. of filtrate was taken and heated with 10 c.c. of conc. HCl for 90 min. on a boiling water-bath. The liquid was then evaporated to 5 c.c. *in vacuo* and the rest of the acid and water removed by washing the flask down twice with absolute alcohol, and taking to dryness *in vacuo* each time. The rest of the procedure was as originally described. Each batch of crude leukotaxine and its fractions were extracted in the same manner.

The effect *in vitro* of the trypan blue and neoantergan on histamine itself and on the biological assay was tested by adding 150 μg. histamine acid phosphate to 0·5 c.c. of each solution and then putting the solution through the histamine extraction procedure.

The extracts were first tested on a piece of guinea-pig's ileum suspended in 2 c.c of Tyrode's solution containing atropine (0·1 μg./c.c.) in comparison with a standard solution of histamine acid phosphate (The British Drug Houses, Ltd.). In some cases, at the end of the experiment a small amount of neoantergan (*N*-*p*-methoxybenzyl-*N*-dimethylaminoethyl-α-aminopyridine acid maleate), 0·005–0·01 μg., was added to the bath. After this treatment the preparation became temporarily insensitive to small doses of histamine, but remained sensitive to other stimulating substances. When the preparation was desensitized to histamine in this way it also became equally insenstive to the extracts. Most of the extracts were also tested in comparison with histamine by intravenous injection into atropinized cats, anaesthetized as described above. Concentrations of histamine present were calculated in terms of histamine base. Blood-pressure readings often gave lower estimates of the histamine content than those obtained with guinea-pig's gut. The discrepancy was never more than about 50% and usually much less, and may have been due to experimental error. The results of all tests were in general agreement, and there can be little doubt that histamine was the main active principle.

Since the injury may alter the size and weight of the skin, results were calculated on the basis of the areas originally mapped out for treatment.

Histological examination. In some cats vitally stained with trypan blue, strips of normal and treated skin were excised, fixed in Susa fluid and cut in serial paraffin sections. The haemalum-eosin method and van Gieson's method were used for general microscopic examination. The contrast staining of the skin macrophages was carried out with aqueous solution of safranin.

RESULTS

'Trypan blue' and 'neoantergan' tests in vivo

The first method used by Menkin (1936) to determine the effect of leukotaxine on capillary permeability consisted of injecting the cell-free exudate intracutaneously in rabbits. This was almost immediately followed by intravenous

injection of 10 c.c. of 1% trypan blue in saline per rabbit. The extent of accumulation of the dye in the treated area of the skin served as a gauge of the result.

In cats treated intradermally with different concentrations of histamine it was found that the effect depended on the dose of histamine and on the tonicity of the injected fluid. The results showed that 10^{-7} and 10^{-6} histamine in saline (i.e. 0·025 and 0·25 μg. per injection) had no effect, but 10^{-5}, 10^{-4} and 10^{-3} histamine (i.e., 2·5, 25 and 250 μg.) induced a definite and progressively more intense staining of the cat's skin. Trypan blue injected intravenously into the cat entered almost immediately the site of the skin treated with leukotaxine, histamine and moderate heat, so that the corresponding areas soon became royal blue in colour in contrast with the cyanotic appearance of the rest of the skin. After 1–5 hr. the picture became more or less variegated because the dye accumulated in the normal untreated skin as well. After 12–24 hr. the whole skin took on an uniform blue colour, having a porcelain-like appearance, except the areas of the burned skin in which the deep blue colour was still in marked contrast to that of the remainder of the skin.

The intradermal injection of any of the leukotaxine preparations induced an almost immediate blue reaction which followed approximately the same pattern as that induced by about 2·5–25 μg. of histamine. Histamine assays on the crude leukotaxine preparations showed the presence of 20–50 μg. histamine/g., while exudate leukotaxine was found to contain 8–10 μg./g. The histamine (Figs. 1 and 2) was transferred almost quantitatively to the alcoholic extracts, the alcohol-insoluble fractions being found to contain, at the most, only traces (< 1 μg./g.) of histamine.

Although some of the preparations of leukotaxine were thus found to contain histamine, the presence of the latter is not considered to be the cause of the effects for the following reasons. The amount of histamine required to produce the effect was four–ten times as great as the amount estimated to be present even in the alcohol-soluble fractions (30–70 μg./g.). The alcohol-insoluble fractions also gave a positive response in this test although they contained no detectable histamine.

When the leukotaxine preparations were boiled with conc. HCl for 90 min. they lost their effect on the skin, although their histamine content was unaffected by this treatment.

Neoantergan was found to antagonize the effects of histamine and leukotaxine on the skin capillaries as demonstrated by the trypan blue test. The results showed that 10^{-4} neoantergan (i.e. 25 μg. per dose injected) produced no reaction, but when injected intradermally 15 min. before histamine was able to abolish entirely the effect of 2·5 μg. and partially the effects of 25 and 250 μg. of histamine. The antagonistic effect of neoantergan on the blue reaction induced by both fractions of leukotaxine was also observed in cats, and, to

a less extent, in rabbits. In some cases the blue reaction was completely abolished, but more often it was only diminished.

Some effects of trypan blue on cats and on the guinea-pig's ileum

Fig. 1 shows that the intravenous injection of 1% trypan blue in saline decreases slightly the blood pressure of the cat, but is without effect on the

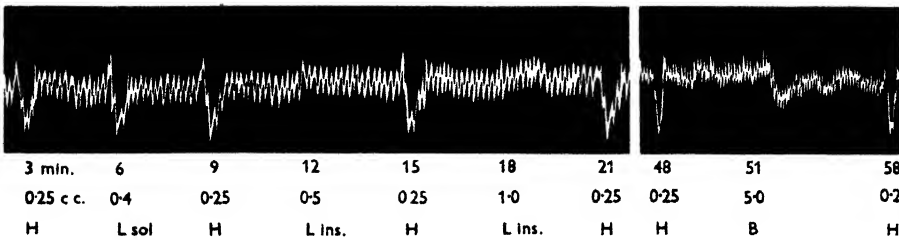


Fig. 1. Effect of leukotaxine and trypan blue compared with that of histamine on the cat's carotid blood pressure (anaesthetized and atropinized with both vagi cut in neck). H = histamine (0·25 c.c. ≡ 0·5 μg. of histamine base). L sol. = 3% alcohol soluble fraction of fibrin leukotaxine; L ins. = 3% alcohol insoluble fraction of fibrin leukotaxine; B = 1% trypan blue.

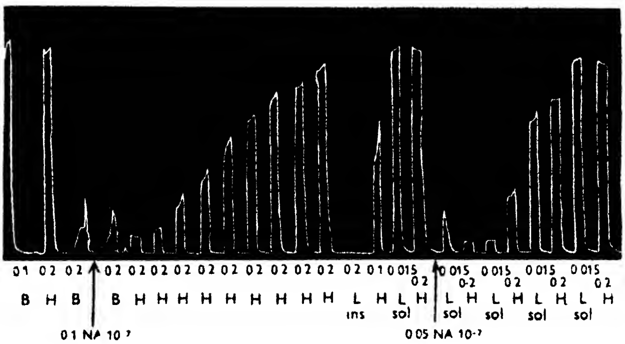


Fig. 2. Effect of leukotaxine and trypan blue compared with that of histamine on the atropinized guinea pig's ileum. H = standard solution of histamine acid phosphate (0·2 c.c. ≡ 0·04 μg. of histamine base); B = 1% trypan blue; NA = neoantergan solution; L sol. = 3% alcohol soluble fraction of fibrin leukotaxine; L ins. = 3% alcohol insoluble fraction of fibrin leukotaxine.

cat's blood-pressure test for histamine. It also causes a slight increase in the extractable histamine of the normal skin, as is shown in Fig. 3. The extractable histamine equivalent in the normal skin of cats treated with trypan blue rose to 17·7 from the average control amount of about 10·5 μg. per outlined area of skin, the maximum value being reached within 1 hr.

The effect of trypan blue was also studied on a strip of the guinea-pig's ileum suspended in 2 c.c. Tyrode's solution containing atropine (0·1 μg./c.c.). In some cases the guinea-pigs had been treated subcutaneously with trypan blue (100–200 mg./kg.) 6, 9, 12 and 15 days before being killed. Fig. 2 shows some

stimulant effect of trypan blue on the gut. This effect seems to be a direct one and not due to formation of histamine in the gut because neoantergan added to the bath did not abolish the effect of trypan blue, although the preparation became temporarily insensitive to small doses of histamine and leukotaxine (alcohol-soluble fraction). In addition, the response of the gut was rather slow and irregular. These results suggest that trypan blue itself may have some action, both *in vivo* and *in vitro*. In view of these findings it was decided to carry out the experiment on cats treated and untreated with trypan blue.

Comparison of effects of leukotaxine, histamine and moderate burns on skin-histamine

The individual results of these experiments are given in Tables 1 and 2, from the average results of which Fig. 3 has been constructed. The average of the weight of the outlined areas is shown graphically in Fig. 4 in which each point, except for exudate leukotaxine, represents the mean of five observations. The surface area in all cases was the same as in control skin.

The intradermal injection of leukotaxine was found to cause slight congestion of the skin and a little oedema within 1–5 hr. This is true for both alcohol-soluble and alcohol-insoluble fractions, the effect being less marked in the case of the alcohol-insoluble fraction.

The extractable histamine equivalent in the skin rose within 1 hr. to a maximum of 83·5, from the average control amount of about 17·7 μg. per outlined area. This increase in skin histamine after the injection of leukotaxine (alcohol-soluble fraction), in the cat treated with trypan blue, was even higher than that in the burnt area at this time. The high concentration was maintained for 5 hr., but dropped almost to normal within 12 hr. In cats untreated with trypan blue, leukotaxine (alcohol-soluble fraction) induced a smaller increase in histamine equivalent, reaching a maximum value of 43·4 μg. per outlined area within 5 hr. as compared with 21·6 μg. in burnt skin at this time and with about 10·5 μg. in control, normal skin. After 12 hr. this excess histamine fell again to normal, except in those areas treated with exudate leukotaxine where some increase in histamine content was maintained for 24 hr.

Estimates for the areas injected with alcohol-insoluble fractions of leukotaxine and with histamine showed no significant increase in histamine equivalent either in cats treated or untreated, with trypan blue. This absence of significant effect does not necessarily suggest a qualitative difference in the actions of the two fractions of leukotaxine. It is probably due to the fact that the alcohol-insoluble fractions contained less of the active principle. The burnt skin at 60° C. shows, as expected, a large increase in skin histamine equivalent and marked oedema, especially in cats treated with trypan blue, the high concentration and oedema being maintained for 24 hr.

The injection of neoantergan in these experiments abolished or decreased the effect on the skin induced by leukotaxine or by histamine injected intradermally and, at the same time, appeared to reduce the extractable histamine in the skin treated with the alcohol-soluble fractions.

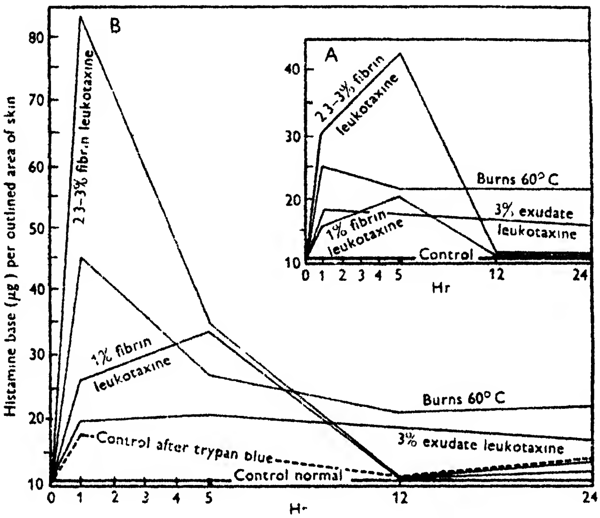


Fig. 3. Histamine equivalent at different times after treatment with leukotaxine alcohol soluble fraction, exudate leukotaxine and heat. A = no trypan blue injected; B = after the injection of trypan blue.

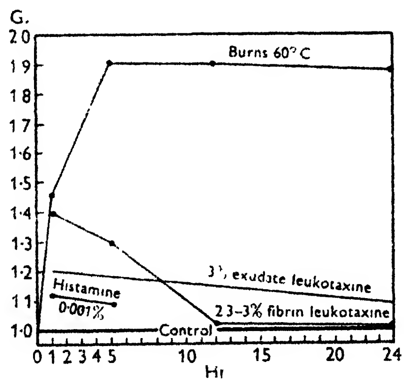


Fig. 4. Effect of leukotaxine, histamine and heat on weight of cat's skin. Treatment at zero time. After moderate burns the weight of the outlined area was almost doubled within 5–24 hr. as compared with control skin. The effects of leukotaxine and histamine were comparatively less and lasted for a shorter time.

Experiments in vitro

The addition of 0·4 μg. neoantergan to the bath (2 c.c.) containing atropinized guinea-pig's ileum made the preparation completely insensitive to histamine for approximately 30 min., the sensitivity returning gradually within the next

2 hr. and more. A hundreth of this amount of neoantergan desensitized the gut to 0·04 μg. histamine for a few minutes only.

TABLE 1. Individual results after trypan blue injection. Histamine equivalents, μg. per area of control skin, burnt skin at 60° C. for 60 sec. and skin treated with various solutions. Tests on guinea-pig's ileum in Roman type. Tests on cat's blood pressure in italics

	Fibrin leukotaxine				Histamine (2·5 μg.)			Controls				
Time after treatment (hr.)	Alcohol soluble	Alcohol soluble NA	Alcohol insoluble	Alcohol insoluble NA		NA	Burnt Skin (60° C.)	Normal	Saline (0·9 %)	Leukotaxine boiled	Neoantergan	Exudate leukotaxine (crude)
1	1 %*		1 %									1 %
	[32·4	24·3	26·7	16·5	24·6	24·3	48·5	26·0		26·4		14·4
	20·3	*26·7*	*16·2*	*16 0*	*13·6*	*20·3*	*23·5*	*11·2*		*13·3*		*13·3*
	2·3 %		2·45 %									3 %
	83·5	57·2	38·0	42·6	43·4	32·2	66·4			33·0		20·1
								13·0	13·3			*20·1*
5	1 %		1 %									1 %
	[46·2	26·7	18 8	19·8	20·0	20·0	33·6	20 0				16·0
	30·8	*20·1*	*12 5*	*11·3*	*13·3*	*11·4*	*21·0*	*13·3*				*13·3*
	26·2	18·5	16·2	16·2	16·2	16·1	22·5	16·1				
	3 %		3 %									3 %
	[38·1		10·9				32·6	14·2			13·9	22·0
	32·0		*10·1*				*27·0*	*13·5*				*20·1*
12	1 %		1 %									
	[12·5		11·0				21·6	12·9				
	7·8		*7·8*					*8·6*				
	[12·5											
	7·9											
	3 %		3 %									
	[11·9		11·1									
	7·8		*7·9*									
	[12·5											
	7·8											
24	1 %		1 %									1 %
	[10·4		12·6				17·6	12·7				16·0
	15·8		*13·1*				*20·0*	*15 9*				*16·0*
	[15·9		15·8									16·0
	10·5		*10·4*									*16·0*
	3 %		3 %									3 %
	[14·8		14·7				30 4	16·0				16·4
	13·6		*14·6*				*28·6*	*16·0*				*13·6*
												[20·5
												16·3

NA = 0·01 % neoantergan injected intradermally 15 min. before the leukotaxine or histamine.
[= Two tests on the same extract.
* = Concentration (w/v) of leukotaxine injected.

Neoantergan (0·4–1·0 μg.) previously boiled with conc. HCl for 90 min. did not affect the sensitivity of the gut to small doses of histamine (0·04 μg.). Larger quantities of neoantergan (10–20 μg.), treated similarly with acid, were found to decrease or abolish the response of the preparation to histamine.

Mixtures of histamine acid phosphate (150 μg.) and neoantergan (500 μg.) were extracted and assayed for histamine. The results showed a 100 % recovery of the histamine added in each of six experiments. The results of six experiments using mixtures of histamine acid phosphate (150 μg.) and trypan blue (250 μg.) showed also 100 % recovery of the histamine. With larger doses of trypan blue (2500-5000 μg.), although added histamine appeared to be recovered, the dye affected the assays, so that the results became inconclusive. The small amounts

of neoantergan and trypan blue present in the outlined areas of cat's skin should not therefore affect the reliability of the results of the histamine assays.

TABLE 2. Individual results without trypan blue injection. Histamine equivalents, μg. per area of control skin, burnt skin at 60° C. for 60 sec. and skin treated with various solutions. Tests on guinea-pig's ileum in Roman type. Tests on cat's blood pressure in italics

Time after treatment (hr.)	Fibrin leukotaxine: Alcohol soluble	Fibrin leukotaxine: Alcohol soluble NA	Fibrin leukotaxine: Alcohol insoluble	Fibrin leukotaxine: Alcohol insoluble NA	Histamine (2·5 μg.)	Histamine (2·5 μg.) NA	Burnt Skin (60° C.)	Controls: Normal	Controls: Saline (0·9 %)	Controls: Leukotaxine boiled	Controls: Neoantergan	Exudate leukotaxine (crude)
1	1 %*		1 %									1 %
	16·0	10·7	10·6	9·1	14·0	10·6	32·9		10·4			10·6
	[16·2	10·2	9·9	9·9	11·4	12·3	16·2	10·2				
	[*14·6*		*8·0*	*8·0*	*8·0*	*9·9*	*12·0*	*9·8*				
	3 %		3 %									3 %
	27·4		14·7				41·0	12·1				18·5
	32·6		20·4					10·4		10·3		18·4
5	1 %		1 %									1 %
	[22·8	12·6	12·5	10·3	12·5	11·2	21·6	10·0				10·6
	[*16·3*	*11·0*	*11·2*	*11·8*	*11·0*	*11·1*	*17·3*	*10·9*			*10·9*	
	21·7	16·4	13·0	16·3	18·5	13·0	19·2		12·8	10·4		
	2·3 %		2·45 %									3 %
	[47·4	13·9	11·6	11·5	11·5	11·4	28·5	10·2				18·2
	[*39·5*	*13·4*	*11·4*	*10·0*	*11·3*	*10·0*	*21·6*	*10·0*				
12	1 %		1 %									
	10·6		10·6				21·6	10·5		11·2		
	3 %		3 %									
	11·5		11·5							10·5		
24	1 %		1 %									1 %
	[10·4		10·5					10·4				10·5
	[*10·5*											
	3 %		3 %									3 %
	[11·4		7·1				22·2			11·3		16·0
	[*12·0*		*8·2*				*20·9*			*12·0*		17·0
	[11·2		7·9									
	[*11·8*		*8·7*									

NA = 0·01 % neoantergan injected intradermally 15 min. before the leukotaxine or histamine.
[= Two tests on the same extract.
* = Concentration (w/v) of leukotaxine injected.

Microscopic changes

Sections cut from the skin treated with histamine could not be differentiated from those of the normal skin. A well-marked migration of polymorphonuclear leucocytes in the areas of skin 24 hr. after treatment with leukotaxine (alcohol-soluble fraction prepared from fibrin) was observed. The excessive crowding with leucocytes was seen in all layers of the dermis and within the small blood vessels. Exudate leukotaxine, obtained from human pus, induced a similar but less intensive infiltration of leucocytes after 1 and 5 hr. After burns, at 60° C. for 60 sec. there was partial heat coagulation of epithelial cells and cellular disintegration in the deeper stratum spinosum. The epidermis became loosened without significant blister formation and there was infiltration of polymorphonuclear leucocytes into deeper layers of dermis. Some macrophages containing phagocytozed trypan blue in various stages of accumulation were apparent in all areas examined, but only 24 hr. after treatment.

These observations are in agreement with earlier results of Grant & Wood (1928) for histamine and those of Menkin (1937) for leukotaxine, and of Duthie & Chain (1939) for protein hydrolysates. The intradermal injection of leukotaxine induces a rapid and increasing migration of polymorphonuclear leucocytes to the site of the injection, but histamine had no such effect.

DISCUSSION

It has generally been assumed that the action of histamine in causing the appearance of a blue area of skin in experiments of the type discussed here is due to a local increase in the permeability of the capillaries. It may, however, be doubted whether this is the correct interpretation of the results. In their early studies of this phenomenon, Rous, Gilding & Smith (1930), Rous & Smith (1931), Smith & Rous (1931*a*,*b*), McMaster, Hudack & Rous (1932), and Smith & Macdonald Dick (1932) found that trypan blue, as well as the more poorly diffusible dye, Chicago blue 6B, appeared first near the venular end of the capillaries and near the venules. They believed that this showed that the permeability of the vessel wall was greatest in this region. Hence these authors concluded that it indicated a structural difference in the permeability of different parts of the capillaries and they criticised the capillary ultra-filtration theory of Starling (1895–6).

On the other hand, Danielli & Stock (1944) are of the opinion that this conclusion is due to a misunderstanding of the experimental results, which are what they would expect on Starling's hypothesis, without assuming any gradient of permeability. The dyes which are classed as poorly diffusible, form colloid solutions and are adsorbed on the plasma proteins, so that they should behave in the same way as plasma proteins. A small proportion (say 1 %) of the dye and protein will leave the blood vessels at the arteriolar end of the capillaries. If the permeability is uniform in different parts of the capillary, a similar small proportion of dye protein will be reabsorbed with the water and other small molecules at the venular end of the capillary, so that the dye and protein will be concentrated in the extravascular fluid in this region, thus producing the observed gradient of intensity of the dye. If this explanation is correct it may be suggested that histamine acts as follows. By dilating the afferent vessels, it raises the intra-capillary pressure and so increases filtration, so that increased amounts of fluid and dye leave the vessels. This fluid is mostly reabsorbed at the venular end of the capillaries, leaving increased amounts of dye in the tissue spaces. After large doses or more prolonged action of histamine filtration is increased still more, and reabsorption is hindered by increased intravascular hydrostatic pressure and increased extravascular colloid osmotic pressure, so that fluid accumulates in the tissues. With the increase in filtration, there is also an increase in lymph flow. If this lymph flow is insufficient to drain away the excess fluid, oedema develops. In this way, it is possible to

explain all the effects of histamine on the capillaries without assuming any action on their permeability.

The substance, or mixture of substances, referred to here as leukotaxine is presumably present in many of the crude preparations of peptone, some of the actions of which are known to be mediated by histamine (skin: Lewis, 1927; Dale, 1929; peptone shock: Dragstedt & Mead, 1937; Gotzl & Dragstedt, 1940; perfused organs: Feldberg & O'Connor, 1937; Rocha e Silva, Scroggie, Fidlar & Jaques, 1947).

The results presented here suggest that the action of leukotaxine in the test with trypan blue is secondary to its effect in increasing the histamine content of the skin. The blue response to leukotaxine was indistinguishable from the blue response to the injection of amounts of histamine such as did appear in the skin. It was similarly inhibited or diminished by neoantergan. It cannot have been due to the presence of histamine in the preparations of leukotaxine, since the quantities present were too small, and since the effect was absent when the preparations had been boiled with acid. On the other hand, the actions of the preparations of leukotaxine on the cat's blood pressure and on the guinea-pig's ileum appeared to be due to the small amount of histamine which was present in these preparations as an impurity.

The effect of leukotaxine on the skin histamine was probably not due to the migration of leucocytes containing histamine into the treated areas of skin, since the skin histamine, together with the oedema and the blue reaction, dropped almost to normal about 12 hr. after the injection, and the excessive migration of leucocytes continued after this time. On the other hand, it is possible that leucocytes concentrated in the vascular bed of skin or migrated into the tissues might liberate their histamine which would then produce the effects. However, such a process would not take place instantaneously, and it should be borne in mind that the blue reaction appeared almost immediately after treatment with leukotaxine, histamine and heat. The effect of leukotaxine on the leucocytes was certainly not due to histamine, since histamine caused no such action.

Trypan blue itself was found to have various effects which complicate experiments of this kind. It caused an increase of the histamine content of skin. It also caused a small fall of blood pressure in atropinized cats and a small contraction of guinea-pig's ileum. The last effect was probably not mediated by histamine since it was not antagonized by neoantergan.

These results suggest that the formation of excess histamine following the intradermal injection of leukotaxine may be responsible for the blue reaction and perhaps the oedema of the cat's skin, and that leukotaxine also induces a more persistent migration of leucocytes. The prolonged blue reaction and oedema in burnt skin are difficult to interpret. Microscopic examination 24 hr. after burning failed to prove either staining of the tissue by trypan blue or any

accumulation of the dye in epidermis or dermis. The only cells which contained granules of phagocytozed trypan blue 24 hr. after vital staining were skin macrophages. The dye seen macroscopically must therefore have been mostly in the extracellular fluid. The concentration of trypan blue at the site of burns and the oedema are probably due, in part at least, to the local and prolonged action of excess histamine.

SUMMARY

1. Small areas of cat's skin were treated with leukotaxine preparations, histamine and heat (60° C.) under anaesthesia and then extracted for the estimation of histamine.

2. After the intradermal injection of leukotaxine, the histamine-equivalent of the skin increased to several times its normal value in 1–5 hr.

3. The intravenous injection of trypan blue caused an increase in the histamine-equivalent of normal skin and enhanced the effect of leukotaxine or of burns.

4. The action of leukotaxine in accelerating the escape of trypan blue from the circulation is mediated by histamine.

5. The known actions of histamine on the capillaries do not necessarily indicate an effect on permeability.

I wish to thank Prof. J. H. Gaddum for his help in planning these experiments and in the presentation of the results, and Dr T. B. B. Crawford for the preparation of fibrin-leukotaxine and for helpful advice.

REFERENCES

Barsoum, G. S. & Gaddum, J. H. (1935). *J. Physiol.* **85**, 1.

Bier, O. & Rocha e Silva, M. (1939). *Virchows Arch.* **303**, 325, 337, 343.

Code, C. F. (1937). *J. Physiol.* **89**, 257.

Cullumbine, H. & Rydon, H. N. (1946). *Brit. J. exp. Path.* **27**, 33.

Cullumbine, H. (1947). *Nature, Lond.*, **159**, 841.

Dale, H. H. (1929). *Lancet*, **1**, 1179, 1233, 1285.

Danielli, J. F. & Stock, A. (1944). *Biol. Rev.* **19**, 81.

Dekanski, J. (1947). *J. Physiol.* **106**, 33.

Dragstedt, C. A. & Mead, F. B. (1937). *J. Pharmacol.* **59**, 429.

Duthie, E. S. & Chain, E. (1939). *Brit. J. exp. Path.* **20**, 417.

Feldberg, W. & O'Connor, W. J. (1937). *J. Physiol.* **90**, 288.

Gotzl, F. R. & Dragstedt, C. A. (1940). *J. Pharmacol.* **74**, 33.

Grant, R. T. & Wood, J. E., Jr. (1928). *J. Path. Bact.* **31**, 1.

Lewis, T. (1927). *The Blood Vessels of the Human Skin and their Responses.* London: Shaw and Sons, Ltd.

McMaster, P. D., Hudack, S. & Rous, P. (1932). *J. exp. Med.* **55**, 203.

Menkin, V. (1936). *J. exp. Med.* **64**, 485.

Menkin, V. (1937). *Arch. Path.* **24**, 65.

Menkin, V. (1938). *J. exp. Med.* **67**, 129, 145, 153.

Menkin, V. (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 103.

Menkin, V. (1940). *Dynamics of Inflammation.* New York: The Macmillan Co.

Menkin, V. & Kadish, M. A. (1938). *Amer. J. Physiol.* **124**, 524.

Rocha e Silva, M. & Dragstedt, C. A. (1941). *J. Pharmacol.* **73**, 405.
Rocha e Silva, M., Scroggie, A. E., Fidlar, E. & Jaques, L. B. (1947). *Proc. Soc. exp. Biol., N.Y.* **64**, 141.
Rous, P., Gilding, H. P. & Smith. F. (1930). *J. exp. Med.* **51**, 807.
Rous, P. & Smith, F. (1931). *J. exp. Med.* **53**, 219.
Smith, F. & Macdonald Dick (1932). *J. exp. Med.* **56**, 371.
Smith, F. & Rous, P. (1931*a*). *J. exp. Med.* **53**, 195.
Smith, F. & Rous, P. (1931*b*). *J. exp. Med.* **54**, 499.
Starling, E. H. (1895–6). *J. Physiol.* **19**, 312.

J. Physiol. (1949) 108, 247–263 612.111.546.32.33

CATION CONTROL IN HUMAN ERYTHROCYTES

By MONTAGUE MAIZELS

From the Department of Pathology, University College Hospital, London

(*Received* 26 *February* 1948)

The accumulation of potassium in animal cells against the concentration gradient has long been a matter for speculation, and it seemed to the writer that the use of a method devised by Harris (1941) might indicate some of the factors concerned in the paradoxical distribution of cations between cells and body fluids.

It is well known that in citrated human blood stored at low temperatures the erythrocytes lose potassium (Dulière, 1931; Drew, Esdall & Scudder, 1939; Downman, Oliver & Young, 1940) and gain sodium (Jeanneney, Servantie & Ringenbach, 1939; Maizels & Paterson, 1940) in accordance with the concentration gradients. Harris (1941) showed that if such cold-stored blood be kept at 37° for a few hours cation distribution tends to return toward the paradoxical levels found in the circulating blood. Repetition of Harris's experiments gave variable results, and it seemed of interest to examine possible factors concerned in the cation movements and especially pH, interdependence of cations, glycolysis and phosphorolysis.

METHODS

A sterile technique was used throughout. Forty-five parts of blood were mixed with 5 parts of trisodium citrate solution (3%) and 1 part of glucose solution (30–50%). 10 ml. of the mixture were centrifuged, supernatant plasma removed and 3 ml. packed cells kept frozen till needed; this was the 'original cell solution'. The residue of the citrated blood was kept 6 days at 4–7°, and then 10 ml. portions were placed in screw-capped bottles containing 1 ml. KCl solution (1·86%) and either acid or alkali. Sometimes the alkali was glycine (0·6 M) plus NaOH (N), and in this case the amount was made up to 1 ml. by the addition of the appropriate amount of NaCl solution (N/2). At other times Na_2CO_3 (N) was used with NaCl solution (N). All plasmas thus had the same initial amounts of Na and K, the former being six to eight times cell Na and the latter about half cell K. To one of the little bottles containing blood neither acid nor alkali was added. This was centrifuged immediately and the packed cells kept frozen; this was the 'cold-storage level' control. All other bottles were kept at 37° for 16–24 hr., after which supernatant plasma was removed, the deposit poured into calibrated haemoglobinometer comparator tubes, corked and centrifuged. Residual plasma was then pipetted off, dregs being washed away with sucrose solution (10%) and 3 ml. of the packed cells decanted with washings into a 15 ml. flask, the volume being made up with distilled water. 'Original' and 'control' cells were treated in the same way. In this cell solution Na, K, phosphate (total acid soluble and inorganic) and glucose were estimated and also the haemoglobin content relative to that of the original cell solution. Details of methods used are described elsewhere

(Maizels, 1943). It will be noted that the alkaline-saline additions to the blood samples were hypertonic, since the addition of iso-osmotic alkali to give the requisite shift of pH involves excessive dilution of the blood plasma. Hypertonic solutions, moreover, check the swelling of cells during storage and so minimize changes in cell and plasma water.

The headings in the various tables require fuller consideration:

(*a*) pH *of cells*. This was measured with a small tubular glass electrode on a fivefold dilution of cells, loss of carbon dioxide being avoided as far as possible. Owing to heavy buffering pH will differ but little from that of undiluted haemolysed cells, but may well differ somewhat from the pH of the intact cell to which, however, it probably bears a consistent relation. In any case, cell pH as set out is the reading taken at the end of a long period of incubation, during which pH is progressively falling possibly by 1 unit or more. Again, bloods mixed with NaOH and NaOH-glycine buffer may both achieve the same final pH, but the former may for some time during the first part of incubation be much more alkaline. This may alter the rate at which cation exchanges proceed during the early stages of incubation, and may also induce early and irreparable cell damage absent in the buffered system. Another factor affecting results is the speed and smoothness of mixing, a slow uneven mix exposing a proportion of erythrocytes to prolonged high alkalinity and causing cell damage which might have been avoided by quicker mixing. For these reasons, therefore, pH figures recorded in a table are merely indices of the relative reactions of the various systems and must be so understood throughout this paper.

(*b*) *Sodium and potassium*. The errors in the estimations are about $\pm 2\%$. To make values comparable these must be referred to the original cell volume. Thus if cold-stored cells have shrunk to 95% of their original volume and their K content is 65 m.equiv./l., K content per 1000 ml. original cells is 62. If after incubation the volume is 110% and K 65, K content per 1000 ml. original cells is 68 and 6 m.equiv. K have entered the cells from the plasma. Changes in cell volume are obtained by comparing haemoglobins in the various cell solutions with that of the original cell solution. The error in this estimation is $\pm 2\%$, giving an overall error of $\pm 4\%$. Concentration of K and Na in cells is obtained by multiplying the cell content (per 1000 ml. cells *final* volume) by 100 and dividing by the percentage of water in the cells; the latter, which is taken as 69·5%, is subject to correction for changes in cell volume due to swelling or shrinkage. Thus, if the water content of the original cells is 69·5%, and if the treated cells swell to 120%, then the water content will rise to 74·5%, or allowing for errors in measuring changes in cell volume, $74{\cdot}5 \pm 0{\cdot}5\%$.

(*c*) *External concentrations of sodium and potassium*. These cannot be clearly defined in relation to the cells; the blood is shaken after cold storage and before incubation. Exchange of ions begins while erythrocytes are fully suspended and continues as the cells settle until at last it is mainly confined to the fully settled but still lightly packed cells and the intercellular plasma. Theoretically, it might seem desirable to keep the blood rocking and the cells suspended throughout incubation, but apart from technical difficulties this was thought undesirable, as constant movement might damage the cells and so alter their permeability. In any case, one is concerned less with a final 'steady state' than with initial external concentrations when ions begin to move with or against their gradients.

In some cases, K and Na contents have been estimated directly on the plasma from the cold-stored unincubated control. Conversion to plasma concentration is effected as follows: plasma from the treated but unincubated controls will vary in volume according to the amount of initial shrinkage or swelling of the cells. It has been found that this varies between $\pm 5\%$, and since plasma volume plus added reagents is 2·3 times cell volume the effect of changes in cell volume on the plasma will be $\pm 2\%$. Water in citrated plasma has been put at $94{\cdot}5 \pm 0{\cdot}7\%$, so that with the experimental errors in estimating Na and K the figures showing plasma concentrations of these ions are subject to an error of $\pm 4{\cdot}5\%$. In other instances plasma Na and K have been calculated from the original haematocrit values, from the known additions of Na and K and from known changes in cell Na and K during storage. Values for K and Na in the original plasma have been assumed to be $4{\cdot}5 \pm 0{\cdot}5$ and 145 ± 5 m.equiv./l. respectively. Figures based on this assumption are subject to an overall error of $\pm 7\%$; they are thus approximate, but afford a measure of the concentrations with or against which cations move.

(*d*) *Glucose*. In some cases blood glucose was measured, but in others only cell glucose. The latter figure does not give an absolute value for glucose metabolized, since glucose used up by the cells will be partly replenished by sugar diffusing in from the plasma, but it does afford an index of the speed of glycolysis in erythrocytes.

RESULTS

pH *and cation movements*

It will be clear from the data in Table 1 that during incubation cations are moving against their gradients, and that the process cannot be explained by any simple physical process but must be ascribed to active transport requiring energy. The actual figures set out, however, are the resultants of an active movement against the gradient and a passive diffusion with the gradient; one must presume that in the cell membrane during incubation are areas devoted to active movement and others through which simple diffusion occurs.

TABLE 1. Cation changes in erythrocytes of stored citrated blood

(Cold-stored 6 days; incubated 18 hr. Plasma concentrations at beginning of incubation: K, 46 ± 4; Na, 164 ± 10 m.equiv./l. water.)

No.	Cell pH at 20°	K content (m.equiv./l.)*	Na content (m.equiv./l.)*	K concentration†	Na concentration†	Inorganic P (mg./100 ml.)*	Acid-soluble P (mg./100 ml.)*	Glucose (mg./100 ml.)
1 CSL	6·98	61	58	85	82	6·6	52·5	312
2	6·62	55	66	72	86	24·1	28·4	295
3	7·00	68	54	98	77	21·7	29·3	300
4	7·28	80	39	116	57	18·7	31·4	210
5	7·38	82	32	124	48	14·8	33·2	—
6	7·51	85	30	134	47	12·3	34·6	154
7	7·84	87	47	140	76	7·3	46·2	118
8	8·40	80	78	117	114	6·9	38·1	170

CSL = cold-storage level of unincubated cells.
* Values at the end of incubation corrected to original cell volume.
† Concentration, m.equiv./l. cell water at the end of incubation.

Between pH 7 and 7·8 K enters the erythrocytes and Na leaves, output of Na and uptake of K being maximal at about pH 7·3–7·4, above and below which point movements are less active. Below pH 6·7 cell Na usually rises above and K falls below the cold-storage value, and somewhere about pH 6·7 passive sodium diffusion and active sodium expulsion balance, a steady state resulting in which it appears as though Na does not penetrate the erythrocyte at all. Above pH 8 passive cation diffusion also exceeds active movement, and again somewhere between pH 7·4 and 8 there is a second point where Na in contrast to K appears to be non-penetrating.

It has been said that the cation movements are most active at pH 7·4, but it must be remembered that this figure is the value at the end of incubation, and the actual optimum may lie anywhere between the initial pH of 8·2 and 7·4. Owing to the large amount of acid liberated during incubation the use of

phosphate or plasma buffer (three parts to one of blood) has not permitted much closer definition of the optimum pH but suggests that it lies between pH 7·8 and 7·3.

Compared with the cold-storage value, total cell base content (Na + K) shows a slight fall below pH 7, up to pH 7·51 and probably up to about pH 7·7, although increased base binding by cell haemoglobin might be expected to lead to a rise in cell base; this point is discussed later. Below pH 7 cell base remains steady or rises slightly, presumably entering the cell in company with citrate which is practically non-penetrating at higher pH. So, too, above pH 7·7 cell base rises, partly because of increased base binding by haemoglobin and partly perhaps because of increased penetration by citrate.

Interdependence of cation movements

Expulsion of sodium during incubation. The question arises whether Na expulsion during incubation is secondary to an active accumulation of K, or K accumulation to a primary expulsion of Na, or whether both are independently active processes. With regard to the first: in normal circulating blood with an active K accumulating mechanism, expulsion of Na is not a logical outcome. Erythrocytes contain haemoglobin and organic phosphate which exert a far greater osmotic effect than all the non-penetrating anions of the plasma. It follows from the Gibbs-Donnan equilibrium that since both Na and K penetrate, these, along with accompanying anions and water, must progressively penetrate the erythrocyte, the cell swelling and finally bursting. This is, in fact, what happens when red cells are placed in solutions of urea or ammonium chloride where lysis appears to be as rapid as in distilled water. In the experiments shown in Table 1, however, two osmotically active non-penetrating external substances have been added: citrate and glycine, the latter in the pH range investigated being largely undissociated. These will oppose the osmotic effects of haemoglobin. If it could be shown that after incubation these substances had entered the erythrocyte to achieve equilibrium, the state of the experimental cells might compare with those in the circulating blood. But in fact at pH 7·4 and after 6 days' incubation, cell citrate was only 5·3% of the external citrate. It was therefore necessary to devise a new set of experiments where heparin replaced the anti-coagulant citrate and Na_2CO_3 the buffered alkali NaOH-glycine. Neither of these replacements are quite satisfactory; omission of citrate by removing an external osmotic attraction leads to increased cell swelling during cold storage, while the unbuffered alkali Na_2CO_3 gives too high an initial pH during incubation, leading to early lysis and also to an ultimate pH of about 7 but rather unpredictable. Further, it was found difficult to achieve a pH greater than 7·1 without marked haemolysis. However, Table 2 shows that even in the absence of external non-penetrating anion K accumulates in and Na is expelled from the erythrocyte.

TABLE 2. Cation changes in erythrocytes of heparinized blood

(Cold-stored 6 days; incubated 16 hr. Plasma concentrations at beginning of incubation: K, 43±4; Na, 196±11 m.equiv./l. water.)

		Contents at end of incubation (m.equiv./l.)*		Concentrations in cell water (m.equiv./l.)	
No.	Cell pH at 20°	K	Na	K	Na
1 CSL	—	82	75	126	115
2	6·20	79	63	120	95
3	6·71	92	45	146	72
4	7·05	95	56	140	83
5	7·19	93	81	138	122

CSL = cold-storage level. Haemolysis slight in no. 4, moderate in no. 5.
* Corrected to original cell volume.

It may be noted that external Na was particularly high because in order to limit the volume of additions normal alkali was used. It was thought that a high external concentration might tend to check cell swelling at least during the cold-storage stage of the experiment. In spite of the steep contrary gradient, however, we have seen that Na passes out of the cell. It follows that expulsion of Na is an active process. Loss of Na could also result from active ejection of water, but in this case cell K would also be expelled unless it were held by some active process.

Uptake of potassium during incubation. It will, of course, be realized that while active intake of K does not imply passive output of Na (since, as we have seen, such a passive output is not compatible with a cell-plasma system freely permeable to cation), it is, on the other hand, possible that active expulsion of Na could lead to a passive uptake of K, against the gradient. It is true that electrical neutrality alone could be satisfied by the simultaneous expulsion of Cl^- along with Na^+, and this doubtless occurs, but such a process would leave unsatisfied the osmotic requirements of non-penetrating cell anion. If then it be accepted that Na expulsion is a primary activity, uptake of K might be either active or passive.

Superficial inspection of Tables 1 and 2 might suggest the former; thus in record 3 of Table 2 cell K has risen by 10 m.equiv./l. and Na fallen by 30; but in record 5, K has risen 11 m.equiv. above the control while Na has also risen 6. It thus appears as though Na and K were now entering the cell together and that K entry is independent of Na expulsion. However, there are several alternatives which do not involve the assumption of active uptake of K, one of which emerges when the exchange of cations is studied at intervals during a period of 24 hr. incubation (Table 3). It will be seen (1 *b*, *d*, *f*; 2 *d*, *e*, *g*) that after 6–8 hr. incubation cell Na is unaltered or else Na flows with the concentration gradient into the cell rising to well above the cold-storage level. This, as we have seen, is only to be expected because of the preponderating effect of non-penetrating cell anion. It is therefore likely that at about 8 hr. Na is

tending towards a simple physical equilibrium as a result of diffusion. Glycolysis is variable in this period, but between 6 and 24 hr. it is invariably active and pH falls by a whole unit or more while Na ebbs from the cell against the gradient, 39 m.equiv./l. being swept away in a few hours (2*e* and *f*). In the first stage K is little affected, but after 8 hr. it begins to enter the cell. It is possible that this entry is passive and compensates for active Na expulsion even though the expulsion is from a very high level to one which is still above the control value. In other words, it is possible that findings at high pH where both Na and K have increased in the erythrocyte at 16–24 hr. have occurred as follows: first Na enters to well above the cold-storage value tending toward a simple physical equilibrium; then Na is actively expelled and K enters passively to satisfy the osmotic requirements of cell haemoglobin and organic phosphate; there will be a stage when K has increased and Na though falling is still above the cold-storage value.

TABLE 3. Effects of pH and time on cation exchanges of incubated erythrocytes

(Cold-stored 6 days. Plasma concentrations at the beginning of incubation: Exp. 1, K 44 and Na 210; Exp. 2, K 38 and Na 198 m.equiv./l.)

No.	Incubation (hr.)	Cell pH at 20°	K content (m. equiv./l.)*	Na content (m. equiv./l.)*	K concentration†	Na concentration†	Inorganic P (mg./100 ml.)*	Acid-soluble P (mg./100 ml.)*	Glucose (mg./100 ml. blood)
1*a* CSL	0	—	82	75	123	112	8·4	—	460
1*b*	6	—	86	70	150	123	—	—	—
1*c*	24	6·71	92	45	146	72	—	—	—
1*d*	6	—	77	85	122	134	—	—	—
1*e*	24	7·03	95	56	140	83	—	—	—
1*f*	6	7·88	76	93	129	158	6·2	—	392
1*g*	24	7·19	93	76	140	116	15·8	—	266
2*a* CSL	0	7·10	77	63	127	104	4·8	48·0	560
2*b*	6	7·74	76	50	150	98	—	—	—
2*c*	24	6·93	92	38	150	62	—	—	—
2*d*	6	8·23	72	64	139	124	3·1	45·9	—
2*e*	9	8·14	70	73	134	140	3·7	49·0	496
2*f*	24	7·17	95	34	157	56	12·7	43·1	356
2*g*	6	8·49	77	67	152	132	—	—	—
2*h*	24	7·26	93	51	160	89	—	—	—

CSL = cold-storage level of unincubated cells.
* Values at the end of incubation corrected to original cell volume.
† Concentration, m.equiv./l. cell water, at the end of incubation.

Once it be accepted that output of Na is active, it becomes difficult to show that entry of K into erythrocytes is not passive, for so long as Na is not available to the cell any factor tending to increase cell base will call on external K. Such factors are:

(1) Active expulsion of Na leading to compensatory rise of K.

(2) Altered physical conditions in the system leading to a call for increased cell base: (*a*) Increase in cell pH. (*b*) A large unbalanced excess of non-

penetrating anion in the erythrocyte leading to a flow of water and salts into the cell. Haemoglobin and phosphate fills this role in normal and heparinized blood. In citrated blood, this osmotic force may be counteracted by adding enough non-penetrating anion (citrate) to the external phase. (*c*) In the case of systems so balanced, subsequent dilution of plasma citrate would cause base to enter the cell. (*d*) Increase of plasma base at any stage of the experiment would cause base to enter the erythrocytes.

(3) Active uptake of K. Before this can be assumed all the preceding factors must be excluded, and this has so far proved impracticable.

An example may make this clearer; blood was cold-stored with a solution containing heparin and KCl and NaCl (60 m.equiv./l. of each). At the end of cold-storage cell K was 73 and Na only 12 m.equiv./l. On incubation owing to the acidity developed cation movement of any kind was slight; little output of Na was possible from this low level, but neither did any rise occur; cell K, however, rose slightly, presumably in response to a call for increased cell base evoked by the osmotic attraction of the excess of non-penetrating anion within the red cell. To balance the latter, part of external KCl was replaced by potassium citrate at the beginning of cold storage, but on incubation this system also became so acid that little cation movement occurred. It was therefore necessary to alkalize the blood, either with strong NaOH which raised plasma base by over 12% or with dilute NaOH which decreased the external non-penetrating anion citrate, results which would in either case direct base into the cell. In one such experiment K rose from the cold-storage value of 73 to 83, while Na fell from 14 to 10 m.equiv./l. Clearly, the fall of Na may account for half the rise in K, and the change in external base or citrate at the beginning of incubation may account for the rest of the rise in cell K, in whole or in part; if the latter, then the possibility of a small active uptake of K remains, but cannot be proved in the presence of so many complicating factors all tending to increase cell base. All that can be said at present is that output of Na cannot be explained by uptake of K which, indeed, it often exceeds, and that such output against the concentration gradient must be an active process. In the case of K, on the other hand, physical processes are present which could account for its uptake against the gradient, and no evidence in favour of active transport has been found though the possibility has not been excluded.

Total base in incubated erythrocytes

The values for total base (Na + K) in cold-stored and in incubated erythrocytes are shown in Table 4. Normal erythrocytes have a total base value between 108 and 124 m.equiv./l. When heparinized and stored in the cold for several days there is a considerable rise in total base due to the fact that gain of Na exceeds loss of K, the value reaching 140 or 150 m.equiv./l. On incubation at high pH cations continue to move with the concentration gradients and

increase in base continues. But at moderate pH (moving from about 8 at the beginning to 7 at the end of 24 hr. incubation) total base shows a marked fall, so that it is less than in the cold-stored unincubated cells by 10 or 20 m.equiv./l. Since the pH of the alkali-treated incubated cells is usually greater than that of the unincubated cells an actual increase in cell base might be anticipated in the former. Thus in Exp. 5 (Table 4), with a rise in pH of 0·47, the base binding

TABLE 4. Total base in cold-stored and incubated erythrocytes

No.	Blood	pH 20°	Contents (m.equiv./l.) K	Na	Na+K	Acid-soluble phosphate
1	U	—	82	75	157	—
	I	6·71	92	45	137	—
2	U	6·94	82	69	151	34
	I	7·00	88	41	129	20
3	U	6·90	82	60	142	31
	I	7·07	95	29	124	27
4	U	7·10	77	63	140	29
	I	7·17	95	34	129	26
5	U	6·91	87	53	140	33
	I	7·38	88	34	122	28
6	U	—	73	65	138	—
	I	7·30	93	36	129	—
7	U	6·85	76	67	143	31
	I	7·30	91	44	135	31
8	U	7·02	83	52	135	34
	I	6·91	97	33	130	26
9	U	6·72	67	68	135	29
	I	7·18	85	39	124	21
10	U	6·67	70	51	121	30
	I	6·82	90	27	117	27
11	U	7·23	61	58	119	26
	I	7·51	80	30	118	22
12	U	7·18	67	43	110	27
	I	7·51	92	22	114	22

Nos. 1–6, heparinized; 6–12, citrated. U = unincubated; I = incubated.

capacity of cell haemoglobin increases by about 20 m.equiv./l., but base actually falls by 18 m.equiv./l. Hence, the fall of base cannot be attributed to any pH change. It could be brought about by a redistribution of some cell anion which is normally non-penetrating, the chief of these being phosphate. Actually, a small loss of total phosphate does occur which is usually negligible when compared with the base loss. This is shown in Table 4, where phosphate is given in milli-equivalents; the figures are approximate and based on data for the equivalence of cell phosphate given by Maizels & Farmer (1939). If, now, it be recalled that at the onset of incubation NaCl and Na_2CO_3 are added to the blood and increase the concentration of plasma base by about 25% and that, in spite of this accession of external base, cell base actually falls, it will be

realized that the output of base on incubation, like the output of Na on which it depends, must be active.

In order to satisfy electrical neutrality cation escaping from the erythrocytes must be accompanied by OH^- or other diffusible anions. Since the cells are not more acid after incubation than at the end of cold storage, it seems that the cations must leave the cells in company with Cl^- and HCO_3^-. Thus, in Exp. 7 of Table 4 18 m.equiv. diffusible anion must leave the cell in company with base actively discharged and about 20 m.equiv. more in association with hydrogen ions in order to free sufficient cell base to permit the observed shift in cell pH from 6·91 to 7·38; it being assumed that 320 g. haemoglobin present in a litre of erythrocytes binds about 40 m.equiv. base for an increase of 1 pH unit (Maizels & Farmer, 1939).

In citrated as in heparinized blood, K and Na move with the gradient during cold storage but the rise in total base is less, possibly because the osmotic attraction of non-penetrating cell anion is opposed by plasma citrate; similarly, during incubation, cell base falls below the cold-storage level, but the decrease is less marked than in heparinized bloods.

The figures in Table 4 are too few to permit of statistical analysis, but they suggest that the higher the rise of base during cold storage, the greater is the fall during subsequent incubation. Thus in Table 4, nos. 1, 2 and 3 have a cold-storage base level averaging 150 m.equiv./l., falling by 20 m.equiv. during incubation; 4, 5 and 6 average 139 m.equiv. with a fall of 12; 7, 8 and 9 average 138 m.equiv. with a fall of 9 m.equiv.; and 10, 11 and 12 have an average cold-storage base of 117 m.equiv., falling only 1 m.equiv. during incubation.

On the basis of the preceding, it is suggested that cells take up base during cold storage, and during subsequent incubation tend to return to a 'setting' of about 125 m.equiv./l., the exact figure depending on experimental conditions. We have little knowledge of how this is achieved. It may be accepted that the further active movements proceed, the greater will be the disturbance of the pre-existing physical equilibrium and the greater the forces opposing further active movements; thus the concentration gradients against which cations have to move grow ever steeper, while the escape of Na from the cells in company with diffusible anion and a corresponding amount of water will concentrate non-penetrating anion (haemoglobin and organic phosphate), and this in accordance with the Gibbs-Donnan equation will increase the physical tendency for base to diffuse back into the cells. Hence, it may be that the so-called setting of the base level is the resultant of the opposed physical and biological forces. However this may be and in the absence of fuller data, it would seem fair to summarize the findings by saying that the higher the rise in cell base during cold storage, the greater the fall during incubation. It is suggested that this adjustment has a physiological value in that it tends to check in the erythrocyte the natural tendency for base to rise as a result of the electrostatic and osmotic

attraction of the contained haemoglobin and organic phosphate—a tendency which is presumably present in all animal cells by virtue of the excess of non-penetrating anion they contain.

Change in cell volume during incubation

Since cold-stored erythrocytes lose base to plasma when they are incubated it follows that loss of water and cell shrinkage must occur if osmotic equilibrium is to be maintained. In the previous experiments plasma base during cold storage was about 165 and 185 m.equiv./l., which as a result of subsequent additions reached a value of 200–240 at the beginning of incubation. It follows that the relevant changes in cell volume were obscured. In Table 5 plasma-base content during cold storage and incubation were kept practically the same—at 175 m.equiv./l. in nos. 1 and 2 and at 164 in nos. 3 and 4, and it will be seen that cell-base content and volume decrease during incubation. The figures may be contrasted with the cold-storage levels (records 1 *a* and *c*; 2 *a* and *b*; 3 *a* and *b*; 4 *a* and *c*) and also with the figures obtained in glucose-free bloods incubated in otherwise identical solutions (records 3 *b* and *c*; 4 *c* and *d*). Here active cation movements are absent; cell K falls and Na rises with the concentration gradients, total base increases and the cells swell. It is possible that the rise of base is due to increased permeability arising from impaired nutrition of the cell in the absence of glucose, and certainly the cells of glucose-free bloods were prone to show slight haemolysis. On the other hand, cells of glucose-free bloods showed no increased permeability during 6 days' cold-storage (records 1 *a* and *b*; 4 *a* and *b*; 5 *a* and *b*), while incubation with glucose-free buffered plasma for only 6 hr. was associated with increase in cell base and volume without haemolysis; further, the cells of some glucose-containing bloods with definite haemolysis still showed very active cation movements with decrease in cell base and volume. It is therefore suggested that increase in cell base and volume in the absence of glucose may be due, at least in part, to the absence of active cation movements.

In Exp. 5, cold-storage and incubation plasmas were both hypertonic, and there was no change in cell volume on incubation with glucose, but neither was there any change in base. However, the experiment shows that on incubation in the absence of glucose increase in cell base and volume were marked (records 5 *d* and *e*).

One other point may be noted: in the earlier series of experiments cell base tended to fall to between 130 and 120 at the end of incubation; in these plasma base lay between 200 and 240 m.equiv./l. In Table 5, the base level after incubation was about 115, and this may be associated with the lower plasma concentration of about 170 m.equiv./l.

Glycolysis and the movement of cations

An example of the association of glycolysis with active cation movements is shown in Exp. 5 of Table 5.

It should be noted that the pH records of Exp. 5 of Table 5 differs from those in Table 3. In the latter, each experiment comprises a series of observations; in each series the *same* amounts of alkali are added to samples of blood which are then incubated for different periods of time, the pH falling with the length of incubation. In Exp. 5 of Table 5, as with the experiments of Tables 1 and 2, each experiment consists of an unincubated control blood and several other samples to which *different* amounts of acid or alkali are added before they are incubated, usually for 18 hr.

TABLE 5. Effects of incubation and glucose on cell volume and cation (Cold-stored 6 days; incubated 18 hr.)

No.	Glucose	Percentage original volume	pH at 20°	K content (m.equiv./l.)*	Na content (m.equiv./l.)*	Inorganic P (mg./100 ml.)*	Acid-soluble P (mg./100 ml.)*	Glucose (mg./100 ml. blood)
1 *a* CSL	+	100	7·14	72	54	—	—	—
b CSL	0	101	7·26	72	55	—	—	—
c I	+	94	7·03	91	24	—	—	—
2 *a* CSL	+	102	7·18	58	65	—	—	—
b I	+	94	7·01	80	32	—	—	—
3 *a* CSL	+	104	7·27	71	48	—	—	—
b I	+	101	7·12	85	26	—	—	—
c I	0	111	7·28	69	63	—	—	—
4 *a* CSL	+	105	7·22	81	42	—	—	—
b CSL	0	105	7·31	80	45	—	—	—
c I	+	100	7·01	94	23	—	—	—
d I	0	114	7·15	82	54	—	—	—
5 *a* CSL	+	89	—	74	42	4·4	48·0	306
b CSL	0	89	—	74	40	10·7	48·0	17
c I	+	96	6·92	80	44	20·0	31·4	179
d I	+	89	7·28	86	29	17·2	38·6	70
e I	0	103	7·22	66	64	21·9	30·8	12
f I	0	97	7·76	55	87	16·6	24·2	15

CSL = cold-storage level; I = incubated.
* Values at the end of incubation corrected to original cell volume.

Harris (1941) is of the opinion that the metabolism of glucose is an important factor in energizing the passage of cations across the red-cell membrane, and this view is supported by the present data, where systems incubated without glucose show little or no accumulation of K and often a definite loss, while expulsion of Na is not seen. Again, even in systems with added glucose, gain of K and loss of Na is least marked at low pH where glycolysis is less active (Table 1 and Exp. 5 of Table 5), most evident at moderately high pH where glycolysis is more active and again less marked where, in the presence of slight haemolysis indicating damage to the cell membrane, glycolysis shows some decrease (Table 1, no. 8). It would therefore seem clear that active transport of cations is closely bound up with glycolysis.

Harris (1941) has attempted to correlate sugar disappearing by glycolysis with the amount of active movement of K but finds the figures very variable; this applies also to the present data, and is not surprising when all the factors

involved are considered. Thus the actual figures for cell K are dependent on the passive movements of Na and K and on the active transport of Na. These will vary considerably with the individual peculiarities of the cells of different bloods, with the speed and smoothness with which alkali is mixed with blood before incubation, and especially with small differences in the temperatures of cold storage and incubation; such differences are unavoidable with small refrigerators or incubators in constant general use, and they will greatly influence the rate of glycolysis, the speed of active movements of Na and of the passive movements of Na and K. Hence it is unprofitable to try to equate glycolysis with K movements unless temperature can be accurately controlled and numerous other variables eliminated.

In spite, however, of the poor correlation between glycolysis and movements of cations it is probable that the two are intimately connected although Harris (1941) considers that some intermediate product of sugar metabolism is responsible for energizing cations rather than glucose itself. Thus, he finds that the cells of blood stored without glucose still show some accumulation of potassium. It is not clear, however, from Harris's experiments that all glucose had in fact been used up before incubation. Moreover, bloods incubated with little glucose, in vessels closed merely with a cotton-wool plug tend to be fairly alkaline, a reaction which favours any active movement of cation that may be possible. In my own experiments with cold storage at 4–7°, glucose indeed largely disappeared and here little or no accumulation of K occurred. Still, it is not impossible that some derivative rather than glucose itself is responsible for activating cations and some of this derivative may persist in erythrocytes after glucose has disappeared. Support for this view might seem to be gained from the observation that although glycolysis proceeds throughout incubation active cation movements are not seen for 6 or 9 hr. (Table 3) at which time it might be presumed that sufficient of the appropriate intermediate substance had been formed. It is possible, however, that the very high pH prevailing during the first half of incubation favours passive diffusion of cations and so conceals any active movements. Certainly, as the pH falls during incubation, the passive factor wanes and the active becomes more evident.

Phosphate and cation movements

In general, the data suggest that active movements of cations vary inversely with the inorganic phosphate, but numerous apparent exceptions will be found. Thus at pH 7·1 where active movements are rapid, inorganic phosphate may appear to be little lower than at pH 6·7 where movements are less, but this is because much of the inorganic phosphate formed in the erythrocytes has leaked out at the lower pH as shown by the low total phosphate (Table 1, nos. 2, 3 and 4). Hence it would probably be more correct to say that active cation movements vary directly with the acid-soluble organic phosphorus, that is, the

difference between total acid-soluble P and inorganic P. Exceptions are met at fairly high pH where inhibition of phosphorolysis is marked while expulsion of Na and uptake of K are less evident (Table 1, nos. 7 and 8). These findings are usually associated with some haemolysis and may be due to increased permeability of erythrocytes at high pH. Again, when cells are incubated at moderately high pH actual synthesis of organic phosphate occurs during the first 7–9 hr. so that inorganic phosphate may be lower than in the original unincubated samples (Table 3, nos. 1 *a* and *f*; 2 *a*, *d* and *e*), yet Na moves with the gradient and no accumulation of K occurs. Hence, the mere occurrence of synthesis is not associated with the active transport of cations. It seems likely that movements of cations require that phosphorylated compounds break down actively, yet not so rapidly as to overtake resynthesis. This would explain the findings at low pH, where with rapid conversion of organic to inorganic phosphate, glycolysis ceases and with it the active transport of cations.

Hydrolysable phosphate in cold-stored blood is best preserved at moderately low pH, disappearing rather more quickly at high or very low pH (Maizels, 1943), but in incubated blood it survives best between pH 7 and 7·5 and disappears very quickly below pH 7. On the whole, correlation between cation movements and hydrolysable phosphate is not good, though it would be correct to say that when hydrolysable phosphate falls below 1·5 mg./100 ml. active transport of cations falls.

If the site at which active movements of cations originate be at the same place in the cell as the sites of the associated phenomena, glycolysis and the synthesis of phosphoric esters, then it must be located in or on the red-cell membrane, for it has been shown that in the erythrocyte with its membrane intact glycolysis is rapid and phosphorolysis slow, while after haemolysis, glycolysis is inhibited and phosphorolysis accelerated (Rona & Arnheim, 1913; Guest, 1932). It may further be shown that the outer face of the red cell is not the site of activity in the following way: intact cells are suspended in a watery haemolysate of the same cells made isotonic with sodium chloride; on incubation it is found that glycolysis is active and phosphorolysis slow in the intact cells, while glycolysis is slow and phosphorolysis active in the cell haemolysate which is in contact with the external surfaces of the intact cells. It may therefore be assumed that glycolysis, ester synthesis and the activation of cation movements are located in the substance or possibly on the inner face of the corpuscle membrane. Support for this view is gained from the work of Brooks (1939), who found that radioactive K before concentrating in the sap of *Valonia* showed intense accumulation in the protein envelope of the cell.

DISCUSSION

It may be said at once that there is no clear knowledge of the way in which human erythrocytes maintain a high K and low Na concentration though suspended in a plasma which is rich in Na and poor in K. The earlier conceptions were purely physical; it was first thought that the distribution arose in some inexplicable manner during the active stage of cell development and was perpetuated in maturity by the cell becoming impermeable to all cations. That this view gained acceptance probably arose from the fact that some permeability experiments were of such short duration that cation penetration was not detected. In others it is possible that a steady state existed; thus at low temperatures cations diffuse passively, at higher, actively, so that at suitable 'room temperatures' these might well balance. pH and time relations might provide further opportunities for steady states. However this may be, there can be no doubt that the theory of erythrocyte impermeability is wrong. This follows from the work of Jeanneney *et al.* (1939) and Maizels & Paterson (1940) on passive diffusion of cations into cold-stored erythrocytes, from the passive diffusion of radioactive isotopes (Cohn & Cohn, 1939; Hahn & Hevesy, 1941), from the active passage of Na out of and K into cold-stored erythrocytes after transfusion (Maizels, 1943), and from the observations of Steinbach and Harris. Steinbach showed (1940) that excised frog's muscle placed in K-free Ringer solution lost K and gained Na, but that if after some hours K in the Ringer fluid was raised to only 10 m.equiv./l., K in the muscle fibre rose to 70 m.equiv., while Na was simultaneously expelled. Then in 1941, Harris demonstrated that active cation movements in and out of human erythrocytes occurred at 37°, and that the processes were activated by glycolysis, either directly or indirectly. Support for these observations is given in the present paper, where it is shown that no active cation movements occur in the absence of glycolysis and not even in the presence of glucose unless the pH is appropriate. Thus if stored blood is brought to pH 8 and incubated Na rises and K falls in the cells in accordance with the concentration gradient and in spite of glycolysis; but later, as the pH falls cations move actively against the gradient. The actual findings are the resultant of active transport and passive diffusion, and it would seem reasonable to assume that certain areas of the cell surface are devoted to the former and others to the latter. Active expulsion of Na is inhibited at low pH and accelerated by alkalis up to pH 7·3 (approx.). It follows that passive uptake of K against the gradient is also hastened by rise of pH, and this finding is comparable to that of Fenn & Cobb (1933–4), who showed that the amount of external K required to prevent loss of K from muscle is less at high than at low pH and to that of Jacques & Osterhout (1933–4), who found a similar pH relation to the entry of K into *Valonia*.

The failure of physical processes to explain the observed cation distribution

in frog's muscle led Dean (1941) to accept the occurrence of active exchange and to propose a 'pump' theory. He suggests that either K is pumped into the cells with a complementary output of Na, or Na is pumped out of the cells with a complementary uptake of K or that both processes occur. It is implicit in his writings that any of these processes acting alone would explain the findings, but in the present paper it is emphasized that the first of Dean's suggestions is not correct, since the presence of a large amount of protein and organic phosphate acting as a non-penetrating anion within cells, while permitting active uptake of K would oppose the passive output of Na.

Indeed, this aspect of the matter seems largely to have been overlooked. Since, as we have seen, the erythrocytes contain a large excess of non-penetrating anion and are permeable to cations, these should, together with anions and water, progressively diffuse into the cells in accordance with the thermodynamic requirements until the cell bursts, and this should be true of all body cells and of any cell without an internal or external structure capable of withstanding osmotic swelling. Since in the case of the body cells Na would tend to enter by diffusion from the plasma, and in the case of single or small groups of cells in sea water Na is again likely to be the chief invading cation, there exists a primary need for cells to develop a mechanism for excreting Na if they are to keep their total base constant and survive. It is therefore interesting to recall that such a mechanism exists even in the non-nucleated human erythrocyte, where during cold storage cell base and volume increase and where during incubation the excess base and water are expelled, the mechanism being so adjusted that the greater the previous rise during cold storage, the greater is the active fall during incubation.

Active excretion of Na, however, does not of necessity imply active uptake of K, and it is difficult to see what primary function such an uptake might subserve: in the absence of any evidence in favour of such active uptake, it is suggested that passage of K into cells is passive and secondary to active output of Na.

If this view be accepted, it becomes possible to explain the various Na/K ratios of the different species, for erythrocytes with an active metabolism will expel a large amount of Na and have a large compensatory uptake of K with a low Na/K ratio, while cells with a low metabolic rate will have a small output of Na and a small uptake of K, with a high Na/K ratio; further, those species with a high Na/K ratio and a low metabolic rate might have a low ratio in muscle and other more actively metabolizing body cells. There is, however, an alternative explanation; the cells of different animal species may all have similar metabolic rates but differ in their permeability to cations. Low permeability would then be associated with a standard active output of Na, a small passive leakage back of Na with a preferential passive inflow of the smaller hydrated-K ion, and a low Na/K ratio; high permeability would have the reverse

effect. But whichever mechanism exists, its end must be the maintenance of a constant level of cell base and water.

But while the assumption of a 'pump' for the active expulsion of Na may explain many of the known findings in cell-base distribution, there is (as Dean remarks) no explanation of how the pump works, and though it is clear that glucose supplies the energy and that Na must adsorb on the inner face of the erythrocyte membrane, be rotated to the outer face and ejected there, further knowledge is completely lacking. The practical outcome of these activities is less obscure; they permit cells to survive in a Na-rich fluid medium without swelling or bursting. Hence, phylogenetically, the mechanisms must be very old, and it is possible that passive uptake of K and possibly Mg, due to active output of Na, may have acquired an extra and later significance by facilitating certain enzymatic processes.

The pathological implications of the foregoing may also be of interest; in prolonged severe acidosis a rise in the Na/K ratio might be expected in the erythrocytes and presumably in all the body cells, while in alkalosis cell Na/K might fall. On the other hand, any shift in cell pH compatible with life might be too small to affect the distribution of cations.

SUMMARY

When blood is stored at 37°, there is an active expulsion of Na from the erythrocytes with uptake of K, the processes being energized by glycolysis. It is thought that uptake of K is passive, though the possibility of an active factor has not been excluded.

When blood is stored in the cold there is progressive loss of K and a faster gain of Na with a consequent increase in the amount of cell base. Incubation in the presence of glucose reverses these cation movements, Na being actively expelled and K regained, in each case against the concentration gradient. Since expulsion of Na exceeds uptake of K, cell base which had increased during cold storage tends to return toward the normal level during incubation. These processes are accelerated by the maintenance of a slightly alkaline reaction during incubation; their result is to restore the physiological Na/K ratio in erythrocytes and plasma and to keep cell base and water constant. In their absence, the erythrocytes and, indeed, any cell unprotected by some external or internal resistant structure would burst because of the osmotic and electrical conditions imposed by its own protein.

My thanks are due to Mr F. V. Flynn (in receipt of a Medical Research Council grant) for assistance in the later stages of this work.

REFERENCES

Brooks, S. C. (1939). *J. cell. comp. Physiol.* **14**, 383.

Cohn, W. E. & Cohn, E. T. (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 445.

Dean, R. B. (1941). *Biol. Symp.* **3**, 331.

Drew, C. R., Esdall, K. & Scudder, L. (1939). *J. Lab. clin. Med.* **25**, 240.

Downman, C. B. B., Oliver, J. C. & Young, I. M. (1940). *Brit. med. J.* **1**, 559.

Dulière, W. L. (1931). *C.R. Soc. Biol., Paris*, **107**, 261.

Fenn, W. C. & Cobb, D. M. (1933–4). *J. gen. Physiol.* **17**, 629.

Guest, G. M. (1932). *J. clin. Invest.* **11**, 555.

Hahn, L. & Hevesy, G. (1941). *Acta Physiol. Scand.* **1**, 347.

Harris, J. E. (1941). *J. biol. Chem.* **141**, 579.

Jacques, A. G. & Osterhout, W. J. V. (1933–4). *J. gen. Physiol.* **17**, 727.

Jeanneney, G., Servantie, L. & Ringenbach, G. (1939). *C.R. Soc. Biol., Paris*, **130**, 472.

Maizels, M. (1943). *Quart. J. exp. Physiol.* **32**, 143.

Maizels, M. & Farmer, S. N. (1939). *Biochem. J.* **33**, 280.

Maizels, M. & Paterson, J. H. (1940). *Lancet*, ii, 417.

Rona, P. & Arnheim, F. (1913). *Biochem. Z.* **48**, 35.

Steinbach, H. B. (1940). *J. biol. Chem.* **133**, 695.

J. Physiol. (1949) 108, 264–269 612.22.02:612.262

THE EFFECT OF BREATHING OXYGEN AT ATMOSPHERIC PRESSURE ON TISSUE OXYGEN AND CARBON DIOXIDE TENSIONS

By H. J. TAYLOR

From the Royal Naval Physiological Laboratory, Alverstoke, Hants

(*Received* 13 *March* 1948)

Lavoisier (1783) carried out autopsies on guinea-pigs exposed for some time to high oxygen tensions and found in particular that the lungs were flaccid, inflamed and engorged with blood. Beddoes & Watt (1796) performed an experiment on a large kitten which was kept for 17 hr. in 80% oxygen. At autopsy they found the lungs a florid red colour and the pleura inflamed. Smith (1899) found that some lung damage occurred in animals which had been exposed to high oxygen pressures and suggested that the changes might represent an attempt on the part of the organism to protect the tissues against excessively high oxygen tensions. Since then many authors have reported similar occurrences. In particular Barach & Richards (1934) found that in chronic pulmonary disease such as pulmonary fibrosis and emphysema there is a progressive and marked increase in the carbon dioxide content of arterial blood. Breathing of oxygen improved the subjective symptoms, despite a continued high level of carbon dioxide in the arterial blood. More recent work by Behnke, Johnson, Poppen & Motley (1935), Becker-Freyseng & Clamman (1939) and Behnke (1940) fully confirm these earlier observations on lung changes caused by the inhalation of oxygen at atmospheric pressure. Campbell (1927*a*, *b*) observed a decrease in breathing by as much as 30% in animals exposed to an oxygen-rich atmosphere. He claimed that this change, combined with a fall in haemoglobin percentage and red blood cell count, was evidence of an attempt on the part of the animal to acclimatize itself to these high oxygen concentrations. The work of Soulie (1939) is of significance in this respect; he found that animals exposed alternately to 24 hr. on oxygen and 24 hr. in normal air, did acquire some resistance to subsequent continuous exposure to oxygen if the intermittent exposures were taken to a point when marked pulmonary symptoms appeared. Campbell (1927*a*, *b*; 1929) reported that inhalation of increased concentrations of oxygen resulted in an increase in calculated

tissue oxygen and carbon dioxide tensions. In the first paper Campbell stated that, unlike most animals, cats could not tolerate prolonged exposure to high oxygen pressure. He suggested that a poison is formed in the lungs.

The present investigation seeks to answer these questions—can convulsions be produced in cats by breathing oxygen at atmospheric pressure, does lung damage occur and if so what is the effect on tissue gas tensions, can evidence be produced to show that animals 'adapt' themselves to high oxygen concentrations?

METHODS

Cats were placed in a glass chamber (dimensions $39 \times 27 \times 28$ cm.) with a wooden lid which could be fixed in position with a compound of putty, agar and glycerine. Cooling was accomplished, when necessary, by a coil carrying cold water, which was attached to the inside of the lid. Oxygen was run in from a cylinder at the rate of about 0·5 l. per min., carbon dioxide being absorbed by a 1·5 in. layer of soda-lime on the floor of the chamber. A piece of perforated zinc separated the animal and the soda-lime. The carbon dioxide concentration during an experiment did not rise above 1 %, and was usually 0·4–0·5 % with the animal in the chamber. The oxygen percentage also remained at 98–99 % except for a short time after the chamber had been opened and closed again. Experiments were made on the effects of continuous and intermittent exposures to oxygen.

A cat received an injection, subcutaneously in the back, of about 40 c.c. of nitrogen. The animal was left for at least 3 hr. in order that equilibrium with tissue gases might occur. Samples were then taken for analysis to determine the normal values for carbon dioxide and oxygen tensions. It was then placed in the chamber and oxygen was passed rapidly at first to displace nitrogen, and then as previously mentioned. Samples of the gas were taken from the cat at intervals varying from 2 to 24 hr., analysed according to the method described by Campbell & Taylor (1935), and the oxygen and carbon dioxide tensions calculated. In order to take samples of the gas the animal was removed from the chamber, and exposed to air for a very short time (1 min. approximately). On reclosing the chamber it was each time flushed rapidly with oxygen for several minutes to ensure a quick return to 100 % oxygen.

RESULTS

Continuous exposure to oxygen

In general it may be stated that the tissue oxygen tension rose rapidly when the animal was first exposed; this was followed by a progressive fall which continued until the experiment was terminated by a convulsion or by the death of the animal. In cases when the animal did convulse the value for the tissue oxygen tension was either in the region of, or less than, the normal value for the animal breathing air. In all cases the carbon dioxide tension rose progressively to a value substantially higher than the normal. Typical results are shown in Fig. 1*a*, *b*. Four cats were treated in this manner.

Cat no. 1. The values of tissue oxygen and carbon dioxide tensions for this cat have been shown in Fig. 1*a*. After 72 hr. exposure, when the carbon dioxide tissue tensions had risen to 77 mm. Hg and the oxygen tissue tension had fallen to 4–5 mm. Hg the animal appeared moribund. It was killed by decapitation. The post-mortem findings showed characteristic intense oedema and congestion. The alveoli and some of the bronchi were filled with fluid and red blood corpuscles. There were petechial spots on the lungs.

Cat no. 2. The values of tissue oxygen and carbon dioxide tension for this cat have been shown in Fig. 1*b*. The animal died after 70 hr. exposure to oxygen. It was not possible to obtain a value for the carbon dioxide and oxygen tissue gas tension during the last 10 hr. of exposure, and so the very high value for carbon dioxide tensions and very low value for oxygen tension shown by the previous cat could not be demonstrated. The values up to 60 hr. exposure, however, agree very well with those of cat no. 1. The post-mortem findings were similar to those found for the previous animal.

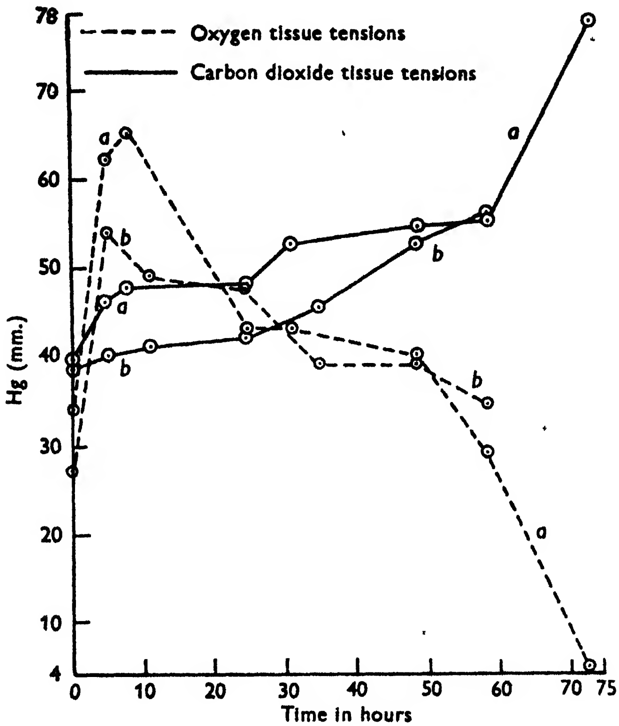


Fig. 1. Effect of breathing oxygen on tensions of oxygen and carbon dioxide in the tissues (*a*) cat 1, (*b*) cat 2.

Cat no. 3. This animal's exposure to oxygen was terminated by a convulsion after 67 hr. exposure. The value of the carbon dioxide tension in the tissues had risen to 69 mm. Hg whilst the value for oxygen had fallen to 33 mm. after having risen to 68 mm. after 4 hr. exposure. When it convulsed the animal was removed from the chamber and allowed to breathe air. After 10 min. the animal again convulsed and continued to do so at short intervals for the next 30 min. (sixteen distinct convulsions). During this time the carbon dioxide tissue tension continued at about 65–69 mm. Hg whilst the value for the oxygen tissue tension

fell to 8·5 mm. Hg. The animal was then killed. The post-mortem findings were similar to those previously described.

Cat no. 4. Showed a very similar result to that given by the previous cat. It convulsed after 53 hr. exposure to oxygen and was allowed to breathe air when it had another convulsion 2 hr. after. The animal survived.

Intermittent exposure to oxygen

In this series of experiments animals were exposed intermittently 24 hr. in oxygen, 24 hr. in air and so on. Two cats (nos. 5 and 6) were used and the results were consistent. On placing in the oxygen chamber the oxygen tissue tensions

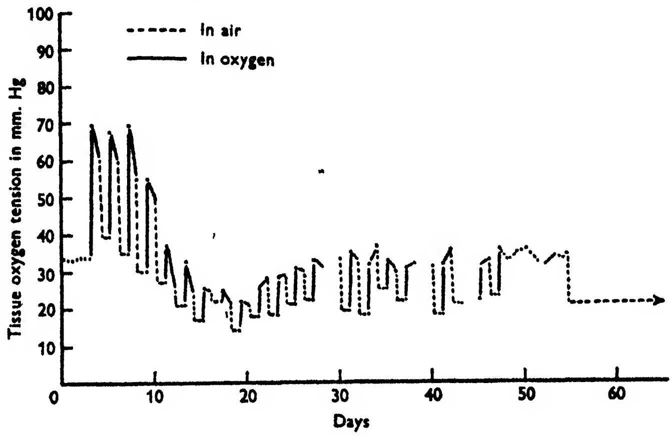


Fig. 2. Effect on tension of oxygen in the tissues of intermittent exposure to oxygen.

rose and remained high at each of three exposures of 24 hr. each, after which the tension dropped rapidly from a value of 70 to 21 mm. Hg in five exposures; the oxygen tension then rose gradually to a value of 33–35 mm. Hg in five exposures at which figure it remained. On the alternate days, when the animal was out of the chamber and breathing air, the oxygen tissue tensions showed a slight rise for three exposures, followed by a fall to 14 mm. Hg in the next five exposures. The tensions then showed a gradual rise in a further 8 days to a value of approximately 21 mm. Hg and remained at this value each time the animal breathed air (see Fig. 2). The values for the tissue carbon dioxide tensions failed to show the progressive rise, associated with continuous oxygen breathing. Tissue carbon dioxide tensions when breathing air are generally somewhat lower than when breathing oxygen, and are close to the normal value of 40 mm. Hg. After 43 days' intermittent exposure to oxygen cat no. 5 was exposed continuously in order to find out if any degree of acclimatization to oxygen had occurred. After 180 hr. exposure there was no significant change in tissue gas tensions and the animal seemed perfectly well. (It will be remembered that the maximum survival time for a normal unadapted cat on exposure to

100% oxygen at atmospheric pressure is 72 hr.) The animal was then removed from oxygen and allowed to breathe air. The tissue oxygen fell to 21 mm. Hg and remained at this value over a period of 21 days. Cat no. 6, after 45 days intermittent exposure, then breathed air over a period of 45 days during which measurements were made of the oxygen and carbon dioxide tensions in the tissues. The results are summarized in Table 1.

Table 1. Tissue gas tensions after acclimatization

Time in days after ceasing oxygen breathing	Tissue CO_2	Tensions O_2
0	41	21
5	43	22·5
26	38	22
45	40	27

As the oxygen tissue tension had shown a tendency to rise again it was thought that the cat might be losing its acclimatization to oxygen breathing, and was therefore made to breathe oxygen, continuously. The time of exposure required to kill the animal was 163 hr. so that some degree of acclimatization was still present. Cat. no 5 when exposed to oxygen continuously, immediately after acclimatization, showed no change in tissue gas tensions, or other ill effects after 80 hr. continuous exposure. It seems therefore that the change producing acclimatization is a slowly reversible one.

The post-mortem findings for cat no. 6 may be summarized as follows: both pleural cavities and the pericardium contained a lightly blood-stained fluid and there was a fibrin clot in the left pleural cavity. Strands of fibrin connected the visceral and parietal pleura. Other organs appeared normal except for the kidneys which were congested, with swollen, prominent glomeruli, whilst the cortex showed fatty streaking suggestive of fatty degeneration.

Experiments using identical exposures on five more cats gave similar results.

DISCUSSION

It has been shown that cats may convulse when breathing oxygen at atmospheric pressure. When this occurs the tissue oxygen tension is in the region of the value for a normal cat breathing air. The convulsion is not due, therefore, to an increased oxygen tension in the tissues, nor is it an anoxic convulsion. The carbon dioxide tissue gas tension is much higher than normal, however, and these findings support the idea that one of the factors causing convulsions is an accumulation of carbon dioxide in the tissues. That carbon dioxide is an etiological factor in oxygen poisoning has been put forward by many authors, and good reviews of the literature have recently been given by Stadie, Riggs & Haugaard (1944) and Bean (1945). The experiments of Campbell (1929), however, provide direct evidence of an accumulation of carbon dioxide in the tissues of animals breathing oxygen at high pressures. The present work shows

that this accumulation occurs in animals breathing oxygen at atmospheric pressure. On the other hand, it seems probable that such accumulation in the present experiments is due to pulmonary permeability, and is interpreted as an interference with the normal removal of carbon dioxide.

If extensive lung damage is prevented by intermittent exposure to oxygen then no marked rise in tissue carbon dioxide tension in the tissues occurs, the pronounced fall of oxygen tissue gas tension is prevented and the animal survives and acquires a degree of acclimatization so that the lethal effect of subsequent continuous exposures to oxygen is arrested. No convulsion occurred under these conditions.

SUMMARY

1. Convulsions occurred when cats breathed oxygen at atmospheric pressure.
2. Breathing oxygen under these conditions led to a progressive fall in tissue oxygen tensions and a progressive rise in tissue carbon dioxide tensions until death or a convulsion resulted.
3. The tissue oxygen tensions of cats exposed intermittently (24 hr. periods) to oxygen and air at atmospheric pressure showed a fall and then a rise to a consistent value, this being somewhat less than the original normal value when the animal was breathing air. Tissue carbon dioxide tensions showed little change.
4. The animals thus treated were acclimatized to subsequent continuous oxygen exposure.
5. This effect is not permanent but it is still present 7 weeks after acclimatization.
6. Evidence is produced to support the view that carbon dioxide is an etiological factor in oxygen poisoning.

I am indebted to Dr C. L. G. Pratt for his continued interest in this work and for much helpful and stimulating discussion.

REFERENCES

Barach, A. L. & Richards, D. W. (1934). *Quart. J. Med.* **3**, 437.

Bean, J. W. (1945). *Physiol. Rev.* **25**, 24.

Becker-Freyseng, H. & Clamman, H. G. (1939). *Klin. Wschr.* **18**, 1382.

Beddoes, T. & Watt, J. (1796). *Considerations on the Medicinal Use and on the Production of Factitious Airs*, 3rd ed. Bristol. (Cited by Bean, J. W. (1945), *Physiol. Rev.* **25**, 24.)

Behnke, A. R. (1940). *Ann. intern. Med.* **13**, 2217.

Behnke, (A. R.), Johnson, F. S., Poppen, J. R. & Motley, F. B. E. P. (1935). *Amer. J. Physiol.* **110**, 565.

Campbell, J. A. (1927*a*). *J. Physiol.* **62**, 211.

Campbell, J. A. (1927*b*). *J. Physiol.* **63**, 323.

Campbell, J. A. (1929). *J. Physiol.* **68**, vii *P.*

Campbell, J. A. & Taylor, H. J. (1935). *J. Physiol.* **84**, 219.

Lavoisier, A. L. (1783). *Soc. Roy. de Médecine*, **5**, 569.

Smith, J. L. (1899). *J. Physiol.* **24**, 19.

Soulie, P. (1939). *C.R. Soc. Biol., Paris*, **130**, 541.

Stadie, W. C., Riggs, B. C. & Haugaard, N. (1944). *Amer. J. med. Sci.* **207**, 84.

J. Physiol. (1949) 108, 270–277 612.492.8:612.627

ACTIVITY AND DRUG RESPONSES OF THE SHEEP UTERUS IN RELATION TO REPRODUCTIVE CONDITION

By N. AMBACHE and J. HAMMOND, Jr.

From the Department of Physiology, University College, London and the Physiological Laboratory, University of Cambridge

(*Received* 7 *May* 1948)

It is well known that the activity, and response to drugs, of uterine muscle may alter with the reproductive condition of the animal. In a number of species there is, for instance, the phenomenon of reversal of the action of adrenaline, which, whilst it relaxes the non-pregnant uterus, in pregnancy causes contraction. It is not always clear from the literature whether the ovaries of the non-pregnant animals investigated contained corpora lutea. Certainly pregnancy is not always normally necessary for such a reversal; in the cow Cupps & Asdell (1944) found this reversal within the oestrous cycle.

On the sheep uterus little work seems to have been done. Polovceva (1940) has made *in vivo* recordings from non-pregnant animals and found the strength of contractions greatly increased at the time of heat, and their frequency diminished. Recently Gunn (1944) has observed, *in vitro*, that adrenaline is motor to the non-pregnant uterus of the sheep but inhibitory at the time of parturition—a reversal in the opposite sense to those previously known.

The results presented here concern the spontaneous activity *in vitro*, and the responses to acetylcholine, adrenaline and posterior pituitary extract, of uteri from a number of ewes killed at various stages of pregnancy, and also from a few non-pregnant animals—some normal, others previously treated with pregnant mare serum gonadotrophin or stilboestrol. It has been found that the action of oxytocin is prolonged during pregnancy in that it is followed (*a*) by the appearance of characteristic 'slow' rhythmic contractions which may persist for as long as 1 hr. after the drug is washed out and which resemble the type of activity exhibited by strips of parturient uteri, and (*b*) by the potentiation of subsequent doses of acetylcholine. It is also shown that the action of adrenaline in pregnancy is a mixture of motor and inhibitory effects and that the inhibitory after-effects may depress the response to other motor drugs.

MATERIAL AND METHODS

Animals. Most of the pregnancy material was obtained through the courtesy of the late Sir Joseph Barcroft, and came from animals killed in the laboratory; other uteri were obtained from animals killed at a slaughterhouse, and were transported to the laboratory in warm Tyrode solution. Fourteen ewes at various known stages of pregnancy, and two ewes in anoestrus (confirmed by examination of the ovaries), were opened up under chloral anaesthesia; on the conclusion of certain observations on the foetus (Barcroft & Torrens, 1946), portions of their uteri were removed for examination. Eight ewes had been kept at the Animal Research Station; seven were anoestrous animals given various hormone treatments before slaughter, and the eighth was killed 24 hr. after a difficult lambing. Eight other uteri were obtained at the slaughterhouse from animals of unknown history. Six were anoestrous and two were in early pregnancy—the stage could be estimated to within a day or two from the weight of the foetus.

Details of hormone treatment are given later: the ovarian response to treatment was not always that intended; lack of animals prevented further investigation of the effects of hormone treatment.

The complete series of animals examined was therefore as follows: *anoestrous*—eight normal, and six with ovulation induced by pregnant mare serum (one each with corpora lutea of about 1 and 3 days old, one with a corpus luteum just beginning to regress, and one in which regression had begun about 3 days earlier; and two given large amounts of stilboestrol, one having a very recent ovulation at the time of slaughter, the other with three well-formed active corpora lutea); *pregnant*—one each at the following stages: 33 days, about 36 and 50 days, 60, 64, 85, 90, 100 (two ewes), 124, 126, and 141 (five ewes) days pregnant and one 1 day after parturition.

Experimental method. Two different types of preparation were used. From most of the non-pregnant, and some pregnant, animals horns were taken. These consisted of the entire top 2–4 cm. of one cornu. From most of the pregnant ewes, and from some non-pregnant, longitudinal strips of muscle were taken. These strips were about ½ cm. wide and of similar length to that of the horn preparations; they were taken from the broadest part of the uterus, in pregnancy from an area between cotyledons.

Preparations were set up in a 10 c.c. 'overflow' type of bath in Tyrode solution (without magnesium), aerated with expired air and kept at about 37° C. A balance type of lever was used. Drugs were added to the bath in a volume not exceeding 0·2 c.c. and were usually washed out after an interval of 30 or 60 sec. The posterior pituitary extract used was pitocin (Parke Davis): though, for convenience, here referred to as oxytocin, this preparation is in fact not free from vasopressin.

The preparations were allowed to remain in the bath for at least 30 min. before any drug was administered. There was no fixed order of drugs, or magnitude of doses, administered to the different uteri; neither was there order in the sequence of uteri examined—experiments were done when the opportunity arose. Once a particular response had been observed in one uterus it was looked for in others; sometimes it was possible to say that in uteri previously examined it was absent; in other cases the procedure necessary to reveal it had not been followed. Though the results are thus not always comprehensive, they seem of sufficient interest for presentation.

RESULTS

Attention must first be drawn to a difference in the behaviour of horn and strip preparations. A typical tracing from an anoestrous horn is shown in Fig. 1; there are fast contractions at the rate of 2–3 a minute. A rhythm of this nature, but weaker and less regular in pregnancy or in non-pregnant sheep with a corpus luteum, was seen in all the horns examined. The non-pregnant strips had a similar rhythm but pregnant strips, with one exception, showed no spontaneous rhythmic activity.

A rhythm of a quite different character (Fig. 2)—slow prolonged contractions, one every 3–4 min., sometimes with a superimposed fast rhythm—appeared in late pregnancy in response to oxytocin, and only once spontaneously when a late pregnant strip (126 days)* was examined after being stored for 2 days in the refrigerator. All other uteri were examined fresh. Gunn (1944) illustrates a spontaneous slow rhythm of similar character in parturient uteri; the fact that it is present in all his tracings may be due to a difference in the anaesthetic.

Fig. 1.

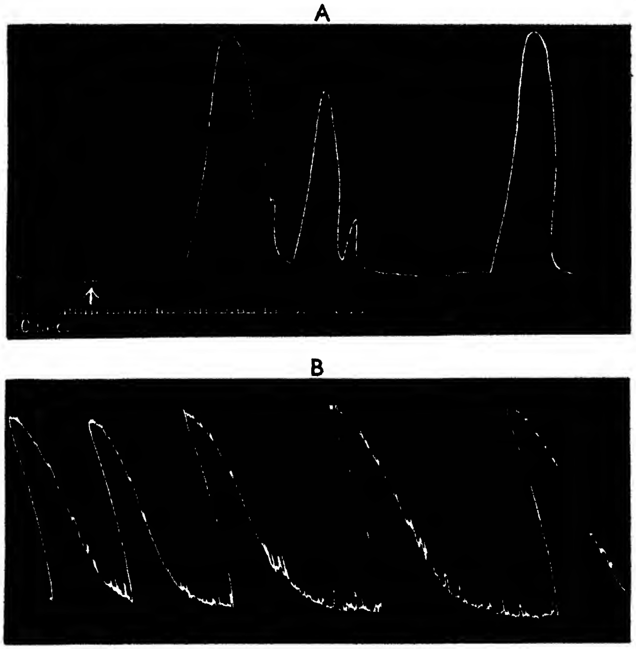


Fig. 2.

Fig. 1. Anoestrous uterus (horn); fast rhythm, and contraction to 10 μg. adrenaline. (For all figures, magnification is $\times$ 10, and time marked at 10 sec. intervals; all tracings read from left to right; volume of bath 10 c.c. throughout.)

Fig. 2. Pregnant strips. A, 141-day strip. Note initial absence of rhythmic activity. At the arrow: effect of 0·001 u. oxytocin; long latent period of 2⅗ min. B, another 141-day strip, showing the slow rhythm 25 min. after a dose of 1 u. oxytocin. The oxytocin was left in the bath for 1 min. only, but the rhythm persisted for over 1 hr.

The anaesthetic did not seem to affect the fast type of rhythm. A horn and a strip from two anaesthetized ewes found to be anoestrous at laparotomy, showed the same sort of activity as did the slaughterhouse material. Horns from three late pregnant ewes (124, 141 and 141 days) also had a fast, though

* This ewe was recorded as 142 days pregnant, but the foetal crown-rump measurement indicated conception at a service a cycle later than the one recorded. Term is normally 147 days, but may be a few less.

weak, rhythm; strips from the same three uteri had a slow rhythm after treatment with oxytocin, but this slow rhythm appeared in none of the horns.

The results from the anoestrous and the pregnancy series will be given first, followed by observations on the individual hormone-treated animals.

Acetylcholine and oxytocin. To both anoestrous and pregnant uteri, acetylcholine (0·1–10 μg.) and oxytocin (1/1000–1 unit) were uniformly motor. The motor action of oxytocin on anoestrous uteri did not remain long after the drug was washed out of the bath. With late pregnant (124–141 days) strips receiving a small dose (1/1000 unit) of oxytocin, there might be a considerable latent period before a contraction appeared (Fig. 2A). With larger doses a much earlier and sometimes prolonged contraction occurred, followed, after washing out, by a slow rhythm. In several experiments where oxytocin had been in the bath for 1 min. or less, the slow rhythm persisted for over an hour despite repeated washes (Fig. 2B). The slow rhythm was seen, twice, to follow on after acetylcholine contractions. Other evidence for a prolonged effect of oxytocin in late pregnancy was an enhancement of the motor action of acetylcholine. This was seen after a small dose (1/1000 unit) had been given for a short time and then washed out, even though it was itself subthreshold (Fig. 3). Carlyle (personal communication) has observed a prolonged action of acetylcholine on sheep uteri treated with oxytocin.

A slow rhythm was not seen, even after oxytocin treatment, in the 33-day pregnant strip, nor in horns at 36 and 50 days (but neither was it present in three late pregnant horns). A strip at 60 days showed a rhythm of 3 min. periodicity after treatment with 1/10 unit of oxytocin, and a rather faster rhythm appeared in the 64-day strip. At 85 and 90 days, and in one at 100 days, no slow rhythm was seen, but it was well marked in the other 100-day strip. A weak slow rhythm (1½ min. frequency) was present in a strip at 24 hr. post-partum. The effect of oxytocin in enhancing acetylcholine contractions was not seen at 33 days, but was observed in both 100-day preparations. The sequence in which drugs were administered to uteri at the intervening stages of pregnancy was such that the effect was not detected.

Adrenaline. The motor effect of adrenaline on the non-pregnant uterus, and its inhibitory action at the end of pregnancy, has been reported by Gunn. However, it is not simply the case that up to a certain stage of pregnancy the drug is motor, and at later stages inhibitory: even in late pregnancy (141 days) adrenaline may cause a uterine contraction. But as pregnancy advances the dose required for a motor effect increases.

Indications have been found of an inhibitory effect in early pregnancy, in that the motor action was usually weakened; in late pregnancy small doses showed a purely inhibitory effect, and the action outlasted the presence of the drug in the bath. When a slow rhythm was present the inhibitory action of adrenaline was shown by the abolition of the rhythm; otherwise it could be demonstrated by testing the response to constant doses of acetylcholine, given before, and at intervals afterwards.

When a large dose of adrenaline had a motor effect in late pregnancy, inhibition followed. Rapid relaxation of the contracted muscle took place

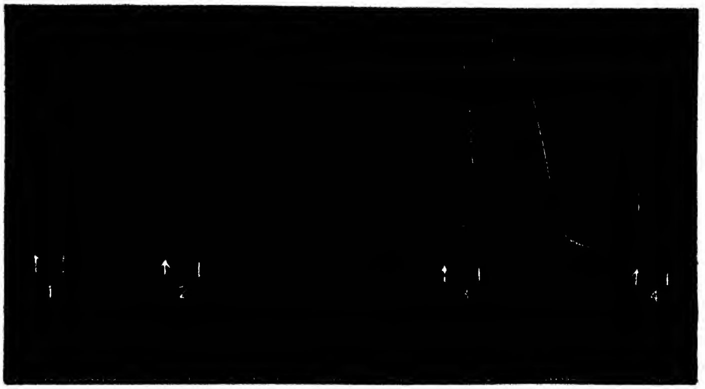


Fig. 3. 100-day pregnant strip; without spontaneous rhythmic activity, and showing the after-effect of oxytocin on the response to acetylcholine. All drugs were left in the bath for 60 sec. (1) Initial effect of 0·1 μg. of acetylcholine; (2) $\frac{1}{1000}$ u. of oxytocin; (3) 8 min. later; 0·1 μg. acetylcholine produced a contraction nearly as large as that previously given by 1 μg.; (4) 200 μg. of adrenaline; immediate contraction and rapid relaxation 30 sec. later—while the adrenaline was still in the bath.

Fig. 4. 141-day pregnant strip; mixed effect of adrenaline. (1) 200 μg. adrenaline produced a contraction, followed by rapid relaxation starting before the drug is washed out. The persisting inhibitory effect is shown by (2) absence of response to 1 μg. acetylcholine (which previously sent the lever off the drum); (3) a second dose of 200 μg. adrenaline has no motor effect.

before the drug was washed from the bath and the shape of the tracing was quite different from that given by a contraction of similar magnitude produced

by acetylcholine. After washing out the bath the motor actions of acetylcholine, oxytocin, and of adrenaline itself, were abolished or reduced for a considerable period. Fig. 4 illustrates some of these features.

In anoestrous horns 1–10 μg. produced large contractions (Fig. 1). At 36 days pregnant, 1 μg. caused relaxation and 10 μg. contraction—both very weak; at 50 days, 1–10 μg. produced no, or weak motor, effects (horn preparations in both cases).

The behaviour of pregnant strip preparations was as follows: At 33 days, 0·1 μg. was motor. At 60 days, 1 μg. once was motor and once (3 min. after a dose of 0·1 μg.) produced no contraction; 10 μg. caused a moderate-sized contraction. At 64 days, 1 μg. caused a small contraction, quickly over, and the weak slow rhythm previously present disappeared. To the 85-day strip a series of doses, at 3 min. intervals, of 0·1, 1, and 10 μg., each left in the bath 1 min., produced no contraction; 3 min. and again 11 min. after the last adrenaline treatment the motor response to acetylcholine (0·1 μg.) was absent; after 18 min. it was present, but depressed. Two hours later 1 μg. adrenaline produced a contraction which finished very rapidly. At 90 days, 10 μg. adrenaline caused no contraction and 35 min. later (when 1 μg. acetylcholine produced good contractions), 100 μg. evoked only a feeble motor effect. At 100 days (two uteri) 1 μg. was ineffective, 10 μg. and 200 μg. respectively gave quickly relaxing contractions. The minimum motor dose of adrenaline was not investigated in late pregnancy, because the long after-inhibitory action would have precluded other observations; a dose of 10 μg. was sometimes effective.

The inhibitory action, and the duration of the after-effect of adrenaline increased with the advance of pregnancy. Inhibitory effects were not seen in the 33-day strip; at 60 days they were probably present but not certainly revealed; they were seen in all later stages.

Hormone-treated animals. These were treated in the anoestrous season with subcutaneously injected stilboestrol in arachis oil or with a commercial extract of pregnant mare serum (P.M.S.) gonadotrophin injected subcutaneously in watery solution. Their uteri were among the first examined, and little emerged from the investigation with drugs. But the uterus from an animal with a 3-day-old corpus luteum, and from another in which the corpus luteum was just beginning to regress, showed rather weak spontaneous activity; in both the motor response to adrenaline was also weak.

Two other animals, treated with rather large amounts of P.M.S. (in unsuccessful attempts to induce multiple ovulations), showed unusual spontaneous uterine activity. Strip preparations were examined from both. Sheep no. 1 was given 1000 i.u. of P.M.S. 12 days before slaughter, and a further 4000 i.u. 5 days later. The ovaries at the time of slaughter contained only a few small follicles, with a regressing corpus luteum—the size and general appearance of which indicated it to have been non-functional for about 4 days. The frequency of the rhythm was of the anoestrous type, but the amplitude of the contractions during a 30 min. period prior to the addition of drugs waxed and waned at 3 min. intervals. Groups of two or three strong beats were succeeded by a number of smaller ones. Adrenaline (1 μg.) caused large contractions. Sheep no. 2 received 1000 i.u. of P.M.S. 11 days before killing and 4500 i.u. 4 days later. With one recent ovulation, and a declining corpus luteum, the ovaries resembled those seen soon after a natural ovulation except that there was more than normal follicle development. This animal possibly came on heat but, as it was not with a ram during treatment, this is uncertain. The strip at first went into a condition of prolonged contraction; later a very strong fast (3/min.) rhythm appeared. These spontaneous contractions were much larger than those seen in any other of the non-pregnant uteri examined. Adrenaline (0·5 μg.) was motor.

Two other animals were given large amounts of stilboestrol and strip preparations were examined from both. Sheep no. 3 was given 1000 i.u. of P.M.S. 12 days before slaughter, 40 mg. stilboestrol 7 days later and a further 20 mg. 3 days after this, 2 days before killing. When killed there was one fresh ovulation, but no trace in the ovaries of any other active or recently regressing corpus luteum. The spontaneous uterine activity was of anoestrous character; 1 μg. adrenaline caused large contractions. Sheep no. 4 was similarly given 1000 i.u. of P.M.S. 12 days before slaughter,

40 mg. stilboestrol 6 days later, with 20 mg. on each of the succeeding 5 days. In this animal there were three full-sized active corpora lutea. The spontaneous activity was weak, but adrenaline (1 μg.) again produced large contractions. 1/1000 unit of oxytocin left in the bath for 7 min. had little effect: after it was washed out the contraction to 1 μg. acetylcholine was much larger than it had been 15 min. earlier. As, however, the response to acetylcholine had previously tended to increase, this result is not definitely attributable to the oxytocin.

DISCUSSION

The changes which occur in the uterus are of interest from three aspects. They may give an insight into the local structures regulating the activity of the muscle; the factors which modify the activity require to be understood; and, since some at least of the factors are probably hormonal, a study of the muscle may reveal the hormone balance in the animal from which the uterus is taken.

The long latent period with small doses, and the prolonged action, of oxytocin in late pregnancy could be interpreted to mean that it acts at a point which it reaches, and from which it is dislodged, with difficulty. The mixed action of adrenaline might be due to an inhibitory action at such a point and the transitory motor effect with larger doses might be produced by an action at a more accessible, though less sensitive, receptor further removed from the contractile element.

Is the slow rhythm seen in pregnant and parturient strips a modification of the fast rhythm of the anoestrous uterus, or is it produced by different factors? If it is a modification, it does not seem to be one made gradually over the greater part of pregnancy. From the same pregnant uterus a horn might show a fast rhythm, and a strip the slow type: as the tip of the horn is relatively much less hypertrophied than is the part from which strips were taken, it may be that changes produced by growth, under the influence of distension, are responsible. Attention must also be drawn to the similarity between the slow rhythm of preparturient strips (Gunn, 1944) and the slow rhythm induced by oxytocin in our experiments.

Progesterone treatment produced adrenaline reversal in the cat (Gustavson & van Dyke, 1931) and desensitized the rabbit uterus to oxytocin (Robson, 1938). The return, in the pregnant rabbit, of a reaction of oxytocin while the corpus luteum is still functional (Knaus, 1927) may be due to an oestrogen-progesterone antagonism, for the growth of the vagina and sterile uterine horn in the latter part of pregnancy (Hammond, 1935) suggest the presence of oestrogen at that stage. It therefore seems likely that both the weakening of spontaneous activity in the presence of a corpus luteum, and the adrenaline reversal, are due to progesterone secretion, and it is possible that, if the sheep resembles the cow in the matter of oestrogen production, the changes found in the latter part of pregnancy are partly caused by oestrogen. Sheep nos. 3 and 4 of the hormone-treated group were given stilboestrol in rather high dosage in the hope that some of the changes seen in late pregnancy might also be found

in them. In that no slow rhythm appeared the results were negative; and, where the action of adrenaline is concerned, it seems (sheep no. 4) that the interaction of oestrogen and corpus luteum was antagonistic.

SUMMARY

1. Uteri were examined *in vitro* from sheep 33 to 141 days pregnant, from normal anoestrous ewes, and from others treated with pregnant mare serum gonadotrophin and stilboestrol. Either the top part of a horn, or a longitudinal strip of uterus was used.

2. A spontaneous fast rhythm (2–3 contractions/min.) was seen in non-pregnant uteri and pregnant horns. The activity was weakened in the presence of a corpus luteum. Pregnant strips were usually inactive; but a slow rhythm (contractions at about 3 min. intervals) normally appeared in late pregnancy after oxytocin had been given. This type of activity was seen as early as the 60th day.

3. In late pregnancy the effect of oxytocin long outlasted the presence of the drug in the bath. The inhibitory action of adrenaline was likewise prolonged in late pregnancy. In spite of its inhibitory action on the pregnant uterus, this drug can cause a motor response as late as the 141st day; the dose required for this effect increases as pregnancy advances, and the contractions produced are of short duration.

4. The few hormone-treated animals gave little indication of the factors responsible for the differences between anoestrous and pregnant uteri.

We are grateful to the late Prof. Sir J. Barcroft for the pregnant uteri, to Mr G. Pluck of the Cambridge Animal Research Station for care of the hormone-treated animals and to the slaughter-house authorities for their co-operation. The work was begun during tenure of a grant (to J. H.) from the Agricultural Research Council.

REFERENCES

Barcroft, J. & Torrens, D. S. (1946). *J. Physiol.* **105**, 22*P*.

Carlyle, A. (1946). Personal communication.

Cupps, P. T. & Asdell, S. A. (1944). *J. Anim. Sci.* **3**, 351.

Gunn, J. A. (1944). *J. Physiol.* **103**, 290.

Gustavson, R. G. & van Dyke, H. B. (1931). *J. Pharmacol.* **41**, 139.

Hammond, J. (1935). *Trud. Dinam. Razvit.* **10**, 93.

Knaus, H. (1927). *Arch. exp. Path. Pharmak.* **124**, 152.

Polovceva, V. V. (1940). *Vestn. Seljskoloz. Nauki Zivotn.* **1**, 127. (Quoted in *Animal Breeding Abstracts* (1942), **10**, 105.)

Robson, J. M. (1938). *J. Physiol.* **92**, 401.

J. Physiol. (1949) 108, 278–281 612.816

OPACITY CHANGES IN STIMULATED NERVE

By D. K. HILL and R. D. KEYNES
From the Physiological Laboratory, University of Cambridge

(*Received* 11 *May* 1948)

The nerve trunk of the walking leg of the shore crab (*Carcinus maenas*) contains many hundreds of non-myelinated fibres which are of a great variety of sizes, most of them being about 2 μ in diameter, but with a few larger fibres up to 30 μ. The overall diameter of the nerve trunk is about 0·5 mm. In appearance the nerve has a whitish opacity caused by scattering of the light, though it is far less opaque than myelinated vertebrate nerve. The experiments described below show that this opacity undergoes a small change when the nerve is stimulated.

Lüthy (1948) has reported that single myelinated nerve fibres undergo a slow, delayed change in their absorption of ultraviolet light when they are stimulated repetitively for 5–10 sec. Flaig (1947) has put forward evidence for there being an alteration in the viscosity of the axoplasm of the giant squid axon when it is stimulated, and Lüthy suggests that the optical change may be caused by swelling or collapse of certain parts of the fibre as a result of this change in viscosity.

Schmitt & Schmitt (1940) failed to detect any change in the birefringence of the giant squid axon during the passage of the nervous impulse.

METHOD

The nerve was illuminated with a nearly parallel beam of white light from a tungsten lamp, the incident beam being either in a direct line with the photocell so that transmitted light was received, or in a slanting direction, in which case no direct light entered the photocell and scattered light only was received (Fig. 1). The intensities of the transmitted and of the scattered light varied in opposite sense in response to a change in the opacity of the nerve. A second photocell was used for balancing the steady component of the light, and the differential photocurrent resulting from a change in the opacity of the nerve was measured as a voltage across a series resistance, using a cathode follower either with visual recording on a long-period galvanometer, or with a cathode ray oscilloscope and photographic recording. The input to the oscilloscope was smoothed with an R.C. circuit of time constant 0·1 sec. to cut down extraneous disturbances.

Owing to the very high amplification required, precautions had to be taken to overcome disturbances caused by transient fluctuations in the light not affecting both photocells equally. Vibrations of the building were reduced by sponge rubber mountings, the disturbances caused by convection of the air in the light beam were overcome by reducing the length of the air path with perspex or glass, and a thick 'bridge' of perspex covered the nerve in its bath to avoid having the light pass through a liquid surface.

The nerve was mounted in a Petri dish of oxygenated *Carcinus* Ringer, with its ends pulled up into a layer of paraffin oil, so that stimuli could be applied and action potentials recorded: it generally remained excitable for several hours. The part of the nerve in the light beam was at least 5 mm. from the point of stimulation.

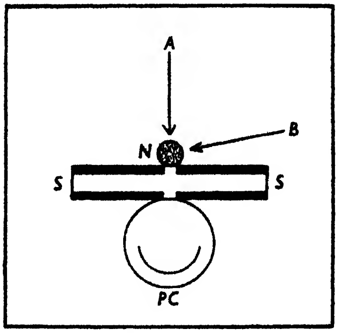


Fig. 1. The nerve N, shown in cross-section, is illuminated by a beam of white light, either directly (arrow A), or obliquely (arrow B). The transmitted, or the scattered light (as the case may be) passes the parallel slits, S, before entering the photocell, PC. Not to scale. Diameter of nerve, about 0·5 mm. Spacing between slits, about 4 mm.

RESULTS

With the smoothed output the effect of a single impulse could just be detected, but it was excessively small. It was possible to obtain quite good results from as few as 20 impulses, but the nerve was generally stimulated at a frequency of 50/sec. for 5 sec. The result of one experiment is shown in Fig. 2. The nerve always became more opaque while the stimulation was proceeding, and the opacity increased for a second or two *after* the stimulus. The first phase was followed by a decrease in opacity. The sizes of the two changes varied greatly in their relation to one another, and the time of cross-over varied correspondingly. Sometimes there appeared to be incomplete recovery. A somewhat unsteady base-line tended to exaggerate these variations. That the effect is largely a change in the *scattering* (rather than *absorption*) of the light was shown by its ready reversibility when the direction of the incident light was altered from the direct to the oblique position. The dependence upon wave-length was not examined, and it would in any case have been difficult to investigate, owing to the necessary reduction in intensity when using monochromatic light.

Controls showed that the observed changes were truly a sign of the activity of the nerve, and eliminated the possibility of the effect being an artefact resulting from the stimulating current itself. The simplest test was to make the nerve inexcitable with KCl, or to kill it with chloroform; in either case the effect was entirely absent when the stimulating current was applied. Another control was provided by the demonstration that the optical response was not seen below a certain threshold shock strength, and also that it built up to

a 'plateau' level as the strength was further increased. A comparable opacity change could be produced by passing a direct current of about 0·5 mA. through the inexcitable nerve; but with a current density as high as this it is not surprising that the tissue should undergo some change of opacity. It is unlikely that the effect observed during activity could arise directly from the action currents, since a rough calculation shows that these are not great enough.

The magnitude of the change can be expressed as the ratio of the change in intensity of scattered light to the resting intensity. The initial increase in opacity is about 1 part in 500 for 250 impulses.

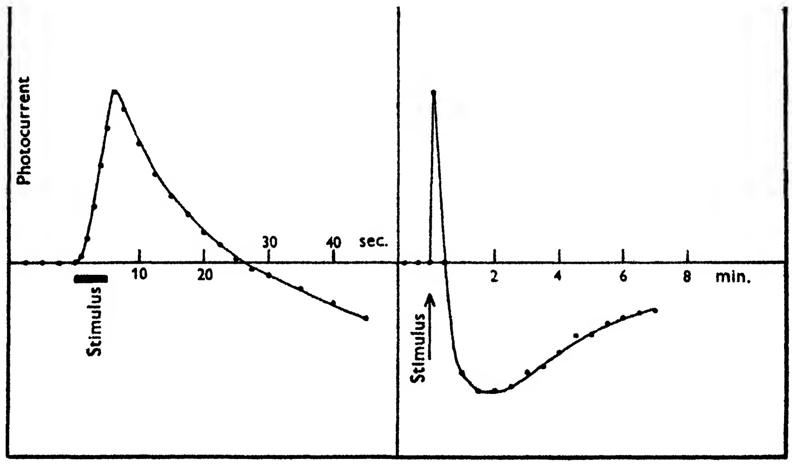


Fig. 2. The opacity change resulting from stimulation at 50/sec. for 5 sec. Oblique illumination: photocell measures *scattered* light. Positive direction represents an increase in opacity. The early phase, shown on the left, was recorded photographically. Temperature, 15° C.

The sciatic nerve of the frog showed no detectable opacity change even following prolonged stimulation at 200/sec. The heat production per impulse of frog's nerve is about 50 times smaller than that of crustacean nerve (Hill, 1932), and the active process responsible for propagation appears to be confined to the nodes of Ranvier (Huxley & Staempfli, 1948). Even so, it is perhaps surprising that nothing was found; but the great scattering of light by the myelin sheath may have masked changes in the opacity of the active elements.

An attempt was made with high-frequency recording, with the smoothing circuit removed, to determine whether any change was detectable *during* the passage of single impulses, but this was unsuccessful owing to interference by 'noise' from the photocell.

DISCUSSION

There are several possible reasons why light may be scattered by the nerve, and one of the first questions that should be asked is whether the change on stimulation takes place in the axoplasm of the fibres or whether, on the other

hand, it is caused by some alteration in the optical properties of the membrane. Flaig (1947) has shown that the viscosity of the axoplasm of the squid fibre appears to increase when the fibre is stimulated and suggests that a partial sol-gel transformation takes place. If this is so it might account for the initial increase in opacity. Krishnamurti (1929) has shown an increase in scattering during the gelation of an agar solution: the clear solution becomes opalescent on setting.

High speed recording has not yet revealed full details of the time course of development of the effect. The opacity increases for a time after the period of stimulation, and the response is evidently not entirely developed during the action potential phase, but at present it is not possible to say more than that.

It is proposed to do further experiments with the giant axon of the squid. In a fibre as large as this there should be a better chance of determining whether the effect has its origin in the axoplasm or whether it is a membrane phenomenon. The variability of the results with crab's nerve is attributable to its great variety of fibres, some of which are more resistant to fatigue than others, and the squid fibre should behave with greater consistency. On the other hand, if it is due to a property of the membrane, the change may be much smaller in the squid nerve than it is in the crab's nerve, the latter having a much greater surface to volume ratio.

SUMMARY

1. An increase in the scattering of white light, followed by a decrease, has been recorded in the limb nerve of the shore crab (*Carcinus maenas*) as th result of stimulation.

2. It has not been possible to determine whether there is any effect coincident with the passage of the impulses. The change has been recorded only as the after-effect of a rapid succession of stimuli, generally 250 impulses in 5 sec.

3. The first phase (increased opacity) reaches its maximum a few seconds after the end of the stimulus; the second phase (decreased opacity) has its maximum at about 2 min. and recovery takes 10-20 min. There is considerable variability in the ratio of the magnitudes of the two phases, and the time course of the change is correspondingly variable.

We are indebted to the Rockefeller Foundation for a grant which has made this work possible.

REFERENCES

Flaig, J. V. (1947). *J. Neurophysiol.* **10**, 211.
Hill, A. V. (1932). *Chemical Wave Transmission in Nerve.* Cambridge.
Huxley, A. F. & Staempfli, R. (1948). *Helv. physiol. pharmacol. Acta*, **6**, C 22
Krishnamurti, K. (1929). *Proc. Roy. Soc.* A, **122**, 76.
Lüthy, H. (1948). *Helv. physiol. pharmacol. Acta*, **6**, C 28.
Schmitt, O. H. & Schmitt, F. O. (1940). *J. Physiol.* **98**, 26.

J. Physiol. (1949) 108, 282–291 612.833.94

OBSERVATIONS ON THE ACTION OF PROSTIGMINE ON THE SPINAL CORD OF THE CAT

By I. CALMA

From the Department of Physiology, University of Liverpool and the Department of Physiology, Middlesex Hospital Medical School, London

(*Received* 22 *May* 1948)

In a previous paper (Calma & Wright, 1947) a technique for the intrathecal injection of drugs in the cat was described and the actions on the spinal cord of eserine injected by this route were reported. In view of the advantages of this technique—the possibility of limiting the action of drugs to a region of the neuraxis and the absence of complicating effects on other organs or systems owing to the slow absorption of substances from the spinal theca—the method of intrathecal administration has been used to study the central actions of prostigmine, a drug which in the hands of previous investigators has behaved rather capriciously. Thus Schweitzer & Wright (1937*a*, *b*) and Schweitzer, Stedman & Wright (1939) found that prostigmine, in common with other quaternary anticholinesterases, inhibited the knee jerk when injected intravenously in the chloralosed cat, and on the basis of these results and of those obtained with eserine and other tertiary anticholinesterases, put forward a theory according to which acetylcholine would have different central effects depending on its intra- or extracellular point of action. Their results with eserine, however, could not be confirmed in the dog by Merlis & Lawson (1939), and according to Bulbring & Burn (1941) the actions of eserine and prostigmine on the isolated perfused spinal cord of the dog are very similar, both drugs causing depression of the knee jerk and potentiation of the flexor reflex. In man, on the other hand, the intrathecal injection of prostigmine decreases muscle tone and reflex excitability according to Kremer, Pearson & Wright (1937) and Kremer (1942). There is evidence in the literature that some of these discrepancies are due to differences in the experimental technique used. For this reason, and in view of the earlier observations with eserine, it seemed necessary to compare the central actions of prostigmine and eserine under the same experimental conditions.

METHODS

Spinal reflexes were elicited at regular intervals in cats under chloralose anaesthesia or decerebrated following the technique already described (Calma & Wright, 1947), with the single difference that in some experiments pulses from a square-wave stimulator were used for nerve stimulation instead of break shocks from a coil. Prostigmine was injected through a cannula inserted in the spinal theca at S.1. Doses refer to the methylsulphate salt of prostigmine.

RESULTS

Control experiments. As stated in the earlier paper the injection of fluids into the spinal theca may cause muscular contractions of an irregular character. These effects produced by the intrathecal injection of any fluids, including saline, are probably due to mechanical excitation of the spinal cord and are generally finished 1 min. after the injection. The interval between the injection of prostigmine and the beginning of its central effects is generally much longer than the duration of these non-specific reactions. It has therefore been possible to distinguish in every case between the two kinds of event.

The possibility that prostigmine may be absorbed from the spinal theca in such quantities as to affect muscular contractions by a peripheral action at the motor end-plates, was investigated by recording simultaneously with the reflex contractions the twitch of the gastrocnemius muscle stimulated through electrodes on the peripheral end of the cut sciatic nerve. No muscular fasciculations typical of the peripheral action of prostigmine of potentiation of the muscular twitches were observed within a period of some 90 min. from the moment prostigmine was injected. The possibility that prostigmine might have been absorbed in any appreciable amount can therefore be excluded.

Action on the knee jerk. In the chloralosed cat, prostigmine injected intrathecally in doses of 0·2–0·5 mg. caused no change in the height and shape of the knee jerks for a variable period ranging from 2 to 15 min. In most preparations the interval between the injection and the first changes in the reflex contractions was about 9–10 min. In some preparations prostigmine caused only a modest increase in the height of the knee jerk; in others a prolongation of the reflex contraction of the quadriceps muscle, as evidenced by the greater area included in the myographic tracing. In most cases, however, there occurred both an increase of the contraction and the appearance of several small additional contractions towards the end of the relaxation after the main jerk. These effects gradually increased in magnitude and extent and reached their peak 20 or 30 min. after the injection. The action of prostigmine was generally long-lasting: the reflex response may not return to its control magnitude and shape within the experimental period, although there may be a decline in the intensity of the effect.

Fig. 1 shows the result of an experiment in which prostigmine affected mainly the height of the knee jerk. Prior to prostigmine (Fig. 1 A) the myogram

of the quadriceps muscle showed a small hump high up in the relaxation curve followed by a slightly irregular downstroke towards the base line. Twelve minutes after the injection of prostigmine (Fig. 1 B) the knee-jerk contraction was greater and, besides the hump at the beginning of the relaxation, two more humps clearly and regularly appeared in the upper third and at the end of the relaxation of the main jerk. Sixteen minutes after the injection (Fig. 1 C) the amplitude of the reflex contraction had further increased and the first hump occurring at the beginning of the relaxation downstroke was longer, so that the angle between the upstroke and the downstroke of the myogram was greater than in the control period. At 18½ min. (Fig. 1 D) the reflex contraction is further enhanced and a short plateau has developed at the peak of the reflex contraction. It may be concluded that prostigmine has increased the number and/or the frequency of the discharging motoneurones and has caused a prolongation of their reflex discharge.

In other preparations prostigmine caused changes in the height of the knee jerk contraction and in the shape of the relaxation curve of the myogram. In the experiment represented in Fig. 2, previous to prostigmine (Fig. 2 A), the main knee jerk was followed by a small additional contraction with a hump on the relaxation curve. Two injections of prostigmine were then given, each of 0·25 mg., at 9 min. interval. Eleven minutes after the first injection (Fig. 2 B) the height of the main reflex contraction was unaltered, but the secondary additional contraction was becoming greater and its relaxation curve prolonged and more sustained. Four minutes later (Fig. 2 C) both the main and the additional contraction were bigger and the additional appendage occurred earlier in the relaxation. Nineteen minutes after the first injection (Fig. 2 D) the changes just described had become intensified and the relaxation was now interrupted by several small humps. Twenty-three minutes after the first injection (Fig. 2 E), following a greatly increased jerk, three secondary contractions occurred and the myogram line fell towards the base line slowly and gradually in small steps, presumably due to the gradual falling off of individual motor units. Prostigmine, therefore, had increased the duration of the central discharge and had brought about conditions of repetitive central reflex excitation owing to which the knee jerk became clonic in character.

The tendency towards clonus developed in different measure in various experiments and was a striking feature of the action of prostigmine on the knee jerk. An extreme example of this tendency was provided by an experiment in which, 2 hr. after intrathecal injection of prostigmine, the knee jerk had become in every way similar to that of Fig. 2 E. It was found in this preparation that if the quadriceps were put under tension, a single tap on the patellar tendon was sufficient to bring about a continuous series of alternate contractions and relaxations, rhythmic in character and of constant frequency and amplitude, which continued for over 15 min. even if no further taps were applied to the

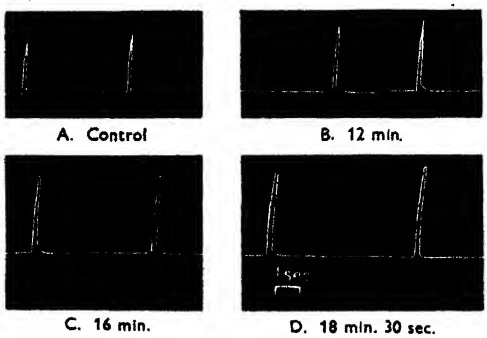


Fig. 1. Cat. 3·1 kg. Chloralose. Record of the knee jerk. A, before prostigmine. B, C, D recorded 12, 16 and 18½ min. after intrathecal injection of 0·3 mg. prostigmine respectively. Drum stopped in intervals between contractions.

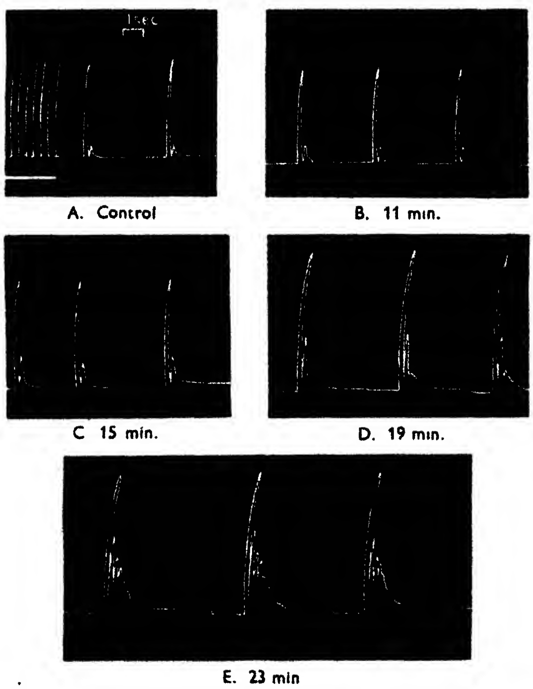


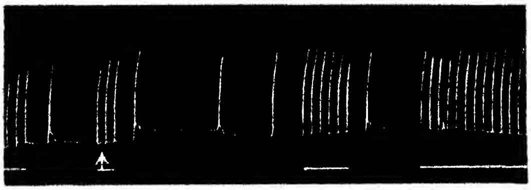
Fig. 2. Cat. 3·1 kg. Chloralose. Record of the knee jerk. A, before prostigmine. In this and subsequent figures the parts of the tracings recorded on slow drum are underlined and the intervals between consecutive contractions represent 10″. For parts on fast drum (not underlined) the time scale is given in each figure but the kymograph was stopped in the intervals between contractions. Between A and B, two injections of prostigmine (0·25 mg. each) at 9 min. interval. B, C, D and E recorded 11, 15, 19, and 23 min. after the first injection of prostigmine respectively.

patellar tendon. Against a background of stretch, prostigmine had caused the appearance of a clonus which through self-re-excitation tended to persist indefinitely. This experiment and others of a similar nature raise the question of the extent to which the effect of prostigmine is conditioned by impulses arising from the reflexly stimulated muscles. The fact that a background of resting muscular tension was necessary in the last-mentioned experiment to elicit a continuous clonic reaction, indicates that proprioceptive impulses from the muscles may play an important part under certain conditions in the re-excitation of the reflexly stimulated motoneurones and may influence considerably the character of the reflex response.

The effects described are certainly due to the central action of prostigmine. This is proved by the fact that muscular fasciculations, typical of the peripheral action of the drug, were absent; the contraction of the gastrocnemius stimulated by shocks applied to the sciatic nerve remained constant throughout the experiment; the blood pressure was well maintained for a long period following the injection of the drug and the frequency of the clonus was dependent on the resting tension of the quadriceps muscle.

Decerebrate cat without spinal block. The intrathecal injections of prostigmine in the decerebrate cat caused the effects shown in Fig. 3. As usual control injection of saline was first made (Fig. 3A). There was an immediate contraction of the quadriceps muscle, and after 35 sec., following a knee jerk, the relaxation was incomplete owing to a slight increase in the tone of the quadriceps, as shown by the elevation of the base line. After 60 sec. the knee jerk had again become stabilized and was inscribed on a steady and horizontal base line. Five minutes later 0·25 mg. prostigmine in 0·5 c.c. saline was injected (Fig. 3B). There was again a contraction of the quadriceps, slightly greater than that produced by saline, and an upward movement of the base line 25 sec. later. Forty-five seconds after the injection the base line had returned to its previous level, at which it was maintained with some irregularities.

Two and three quarter minutes after the injection of prostigmine, the knee-jerk relaxation was incomplete and the subsequent jerk was inscribed on a raised base-line. The following jerks showed similar incomplete relaxations, so that the base-line moved to a progressively higher level with each jerk. The height of the knee jerk decreased as the base line moved upwards. Nine and a half minutes after the injection of prostigmine the base line becomes stabilized at a new high level. The knee jerks inscribed on it were still small and their relaxation was greatly drawn out and incomplete (Fig. 3C). In this experiment, in the interval between two jerks, three wave-like movements of the base line occurred. The action of prostigmine in these decerebrate preparations has been to cause recruitment of the quadriceps motoneurones resulting in the increase of the resting tension of the muscle. Fewer motoneurones are therefore available for the knee-jerk discharge and consequently, as the resting tension increases,

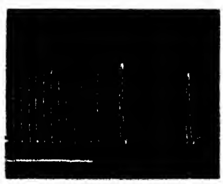


A

B

C. 9 min. 30 sec.

Fig. 3. Cat. 2·7 kg. Decerebrate. Record of the knee jerk. A, at arrow, intrathecal injection of 0·5 c.c. saline. B, at arrow, injection of 0·25 mg. prostigmine in 0·5 c.c. C, 9½ min. after prostigmine. The two white marks show two consecutive knee jerks.



A. Control

B. 13 min.

Fig. 4. Cat. 2·8 kg. Decerebrate. Spinal block at Th. 9. Record of the knee jerk. The kymograph was stopped in the intervals between contractions. A, before prostigmine. B, 13 min. after intrathecal injection of 0·3 mg. prostigmine.

the jerk is progressively occluded. The after-discharge is, however, greatly prolonged even at the maximum of the occlusion.

Decerebrate cat with spinal block at Th. 9. The potentiating effect of intrathecally injected prostigmine on the knee jerk appeared in these preparations as well, with some additional features represented in Fig. 4. Fig. 4A shows the knee jerk in the control period. Prostigmine was then injected and 10½ min. later the contractions began to increase gradually; 13 min. after the injection (Fig. 4B) the myogram shows that the relaxation downstroke fell below the level of the base line and was then followed by an additional small contraction, of greater magnitude than that appearing in the control period. Again the lever fell below the base-line level and then it moved upwards, gradually and in small steps, giving a concave or convex tracing, till it reached the resting level or a slightly higher level, at which it remained with minor irregularities. In this experiment the myogram retained this shape for about 45 min. and then the effect gradually subsided. The novel feature in results of this type is the after-fall of the myographic lever following the main jerk or its appendages or humps. Effects of a similar nature have been observed following intrathecal injection of eserine (Calma & Wright, 1947) and have been interpreted as a long-lasting 'silent period'. It was suggested in the earlier study that this abnormally long silent period may be due to a potentiating effect by eserine of the inhibitory spinal reflexes. The effects caused by prostigmine, being indistinguishable from those of eserine, have probably to be interpreted in the same way.

Action on the crossed extensor reflex. Brief mention only needs to be made of this action because of its general resemblance to that on the knee jerk. In the chloralosed cat, in which the crossed extensor reflex may be absent at the beginning of the recording and during the control period, the intrathecal injection of prostigmine, in the dosage used for the experiments on the knee jerk, caused the reflex contraction to appear, 5–10 min. after the injection. The crossed extensor reflex contraction was initially very modest and increased gradually over a period of 5 or 10 min.; at the same time the relaxation became more prolonged as the effect of the drug developed fully. In this case prostigmine facilitated the reflex and had a 'Bahnung' effect.

The actions of prostigmine on the crossed extensor reflex elicited in the decerebrate preparation with spinal block are represented in Fig. 5B in which it may be clearly seen that they are identical with those already described for the knee jerk in similar preparations: briefly they consist of a more powerful contraction, of a continuous central discharge in the intervals between the impulses and of well-defined periods of inhibition following the reflex contraction. The irregular myographic line in the intervals between two reflexes is not due to muscular fasciculations resulting from a peripheral effect of prostigmine, as it is absent, in the same preparation, from the record of the tibialis anticus.

Action on the flexor reflex. The most frequent effect of the drug was that of increasing the height of the reflex contraction in both the chloralosed and decerebrate preparations. In some cases there was a prolongation of the relaxation curve which fell towards the base line only slowly and gradually. However, these effects were not obtained so regularly as in the case of the knee jerk and crossed extensor reflex. Indeed in some preparations, in which the simultaneously elicited crossed extensor reflex was potentiated, the flexor reflex was inhibited after prostigmine (Fig. 5B). In any case, even in those preparations in which the flexor reflex was strongly potentiated, no tendency to the development of clonus or to the occurrence of a myographic pattern of the type described for the knee jerk and crossed extensor reflex following the contraction of the quadriceps, was ever observed in the myogram of the tibialis anticus.

A Control

B 14 min

Fig. 5. Cat. 3 kg. Decerebrate. Spinal block at Th. 9. In each tracing records from above downwards: left flexor reflex, right crossed extensor reflex. A, before prostigmine. The drum was stopped in the intervals between contractions. B, 14 min. after injection of 0·25 mg. prostigmine.

It may therefore be concluded that prostigmine has a somewhat variable action on the flexor reflex, both potentiation and inhibition occurring after intrathecal administration of the drug.

DISCUSSION

The results just described present several points of interest. In the first place there is a striking resemblance between the action of eserine and prostigmine given by intrathecal injection. Both substances potentiate the reflex

excitability of the spinal cord, both the height of the reflex contraction and the after discharge being affected. Both substances potentiate most regularly reflexes involving the extensor motoneurones and have a more variable action, quantitatively and qualitatively, on the flexor motoneurones. These results resemble on the whole those of Bulbring & Burn (1941), in the sense that eserine and prostigmine are found to have identical actions on the spinal cord, although differing in respect of the knee jerk, and in that the reflex more regularly potentiated in their experiments, the flexor, was least regularly increased in the experiments here presented. These results do not support the theory of Schweitzer *et al.* (1939), although a fairer test of it would be provided by a comparison of the central actions of tertiary and quaternary anticholinesterases of similar chemical constitution, rather than by the comparison of the actions of two such widely different compounds as eserine and prostigmine.

The fact that these drugs have, in spite of their chemical difference, essentially similar actions on the spinal cord, makes it probable that they act by a common mechanism, the most obvious being that of their action on cholinesterase. Persistence of acetylcholine at the synapses would, on this basis, account for the action of these drugs. The prevalent central excitatory effects, and certain small but clear central inhibitory effects (of which the after-fall of the recording lever gives evidence in the case of the knee jerk and crossed extensor reflex, and which have been observed following intrathecal administration of eserine and prostigmine) could therefore be attributed to acetylcholine lingering at the synapses, in accordance with the findings of previous workers (Schweitzer & Wright, 1937 *a, b*; McKail, Obrador & Wilson, 1941) who have clearly indicated that acetylcholine may have a double action, inhibitory as well as excitatory, on the spinal cord although its mode of action is still very obscure (Feldberg, 1945). The divergent results obtained in the case of different reflexes, e.g. the depression of the flexor reflex in some preparations in which the extensor reflexes were simultaneously potentiated and in which the general reflex excitability of the spinal cord seemed enhanced, may depend to some extent on the balance of central excitation and inhibition reflexly produced and on the effects of eserine and prostigmine on such central events.

These results provide evidence of the part which proprioceptive impulses may play in determining the character of the prostigmine effect. It has already been noted that following eserine and prostigmine there develops a tendency to clonic discharge in the extensor, but not in the flexor, motoneurones. Denny-Brown (1929) defines clonus as a series of synchronous rebounds, produced when the reflexly activated motoneurones discharge synchronously, and synchronously emerge from the subnormal period, to be re-excited by the bursts of excitatory proprioceptive impulses arising from the relaxing and stretched muscles. The absence of any tendency to clonus in the flexor motoneurones following eserine or prostigmine may be due to lack of synchroniza-

tion in the flexor motoneurone pool or to the character and nature of the proprioceptive impulse arising from the tibialis anticus.

Lastly there is evidence in the above results of a synchronizing action of prostigmine on the quadriceps motoneurones, of which the clonus is the most clear evidence. As Adrian (1947) has pointed out recently, synchronization occurs under intense excitation of the motoneurones: whether such an effect is due to spreading electrical field (Bremer, 1941) or to nervous impulses conducted along orthodox dendritic connexions cannot be proved on the basis of the findings here presented.

SUMMARY

1. Prostigmine intrathecally injected causes an increase of the reflex excitability of the lumbar segments of the spinal cord in the chloralosed and decerebrate cat.

2. The knee jerk is increased, its after-discharge is prolonged, and there is a tendency to the development of clonic discharges.

3. In the decerebrate cat prostigmine causes an increase of the resting tone of the quadriceps muscle, accompanied by occlusion of the knee jerk.

4. Prostigmine facilitates the crossed extensor reflex in chloralosed preparations and potentiates it in decerebrate preparations, causing a pattern of central discharge very similar to that brought about in the case of the knee jerk.

5. The flexor reflex following prostigmine is generally increased, but in some preparations it is inhibited.

6. There is evidence of a potentiation by prostigmine of some central inhibitory events, such as the prolongation of the silent period in the case of extensor reflexes.

My thanks are due to Prof. W. H. Newton for reading through the manuscript.

REFERENCES

Adrian, E. D. (1947). *Brain*, **70**, 1.
Bremer, F. (1941). *Arch. Inter. Physiol.* **51**, 211.
Bulbring, E. & Burn, J. H. (1941). *J. Physiol.* **100**, 337.
Calma, I. & Wright, S. (1947). *J. Physiol.* **106**, 80.
Denny-Brown, D. (1929). *Proc. Roy. Soc.* B, **104**, 252.
Feldberg, W. (1945). *Physiol. Rev.* **25**, 596.
Kremer, M. (1942). *Quart. J. exp. Physiol.* **31**, 337.
Kremer, M., Pearson, H. E. S. & Wright, S. (1937). *J. Physiol.* **89**, 21*P*.
McKail, R. A., Obrador, S. & Wilson, W. C. (1941). *J. Physiol.* **99**, 312.
Merlis, J. K. & Lawson, M. (1939). *J. Neurophysiol.* **2**, 566.
Schweitzer, A., Stedman, E. & Wright, S. (1939). *J. Physiol.* **96**, 302.
Schweitzer, A. & Wright, S. (1937*a*). *J. Physiol.* **89**, 384.
Schweitzer, A. & Wright, S. (1937*b*). *J. Physiol.* **90**, 310.

J. Physiol. (1949) 108, 292–302 612.741.12

CHANGES IN TRANSPARENCY OF MUSCLE DURING A TWITCH

By D. K. HILL

From the Physiological Laboratory, University of Cambridge

(*Received* 26 *May* 1948)

The first sign of mechanical activity in a stimulated frog's muscle is a transient lengthening (the 'latency relaxation'; Sandow, 1944) which starts at about 2 msec. (18° C.) and reverses into a positive contraction a few msec. later. The heat production of the muscle starts roughly at the same time as the early relaxation (measured at 0° C.; Hill, 1948). The transparency change which accompanies the muscle twitch (Schaefer & Göpfert, 1937; Buchthal, Knappeis & Sjöstrand, 1939) has not hitherto been recorded with sufficient amplification for it to have been possible to discover when it starts, nor the manner in which it does so.

The work described below is concerned with the *earliest* transparency changes following the application of a single electric shock to a frog's skeletal muscle. In addition, brief notes are added in connexion with: (*a*) the long-drawn-out transparency change which *follows* the contraction (von Muralt, 1934*a*, *b*); (*b*) a transient effect on transparency caused by a rapid stretch of the muscle.

Urban & Peugnet (1938) have reported wave-length-specific absorption changes in contracting frog's muscle, and have attributed them to cytochrome *c* and the yellow enzyme. It is not thought that their observations bear much relation to the phenomena described here.

METHODS

When light passes through a muscle it is partly absorbed, but the loss of intensity in the incident direction is largely caused by scattering (Buchthal *et al.* 1939). The first series of experiments was made without attempting to distinguish between these two factors, and a simple optical arrangement was used (Fig. 1 *a*); this was suitable for relating the time of onset of the earliest transparency change with the mechanical response. A further analysis demanded a differentiation between absorption and scattering. The measurement of absorption alone by means of a modified 'ball photometer' (Buchthal *et al.* 1939), although an ideal method in principle, was not practicable with a whole muscle. The arrangement adopted is shown in Fig. 1 *b*. A small telescope, focused for parallel light, was mounted on an arm which could be rotated on a vertical axis. The muscle was held vertically on this axis. A photocell of the 'multiplier' type was fixed to the eyepiece of the telescope. By rotating the telescope it was possible to measure the intensity of the light transmitted

at a series of angles, 0–90°, from the incident direction. The effect of a change of scattering was found to reverse its sign as the telescope was swung round; e.g. an increase in scattering gives a lower transmission in the incident direction but a greater intensity laterally. An effect caused by a change in absorption should have the same sign at all angles.

The frog's sartorius muscle was used. The light source was a 6 V. 36 W. tungsten lamp, overrun at 8 V. to give a high intensity, and supplied by batteries. The light was condensed with a lens to give a parallel beam. The muscle was free in air and kept moist by flowing Ringer solution over its surface between observations; the illuminated patch was about 7 mm. long. In the later experiments with monochromatic light *Farrand* 'interference' filters were used, a set of thirteen of these filters covering the visible spectrum from 400 to 700 mμ.

The output of the photocell passed to a condenser-coupled amplifier, followed by a Mullard E 800 cathode-ray oscilloscope. The mechanical record of the latency relaxation for comparison with the optical events was made with a crystal gramophone pick-up. The muscle was connected with a glass lever, 1 cm. long, inserted in the needle holder of the pick-up, and was tightly stretched to minimize any optical effect due to localized thickening or movement as the contraction wave passed along the muscle.

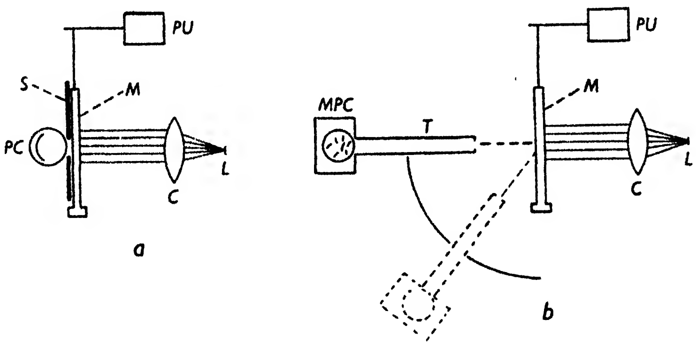


Fig. 1*a*. Simple optical arrangement. Tungsten lamp, *L*. Condenser lens, *C*. Muscle, *M*. Slit, *S*. The photocell, *PC*, is of the single-stage vacuum type. The crystal pickup, *PU*, measures the mechanical response.

Fig. 1*b*. The arrangement for 'directional' recording. *L*, *C*, *M* and *PU*, as in Fig. 1*a*. Telescope, *T*. Multiplier photocell, *MPC*. The long axis of the muscle is actually at right angles to the plane of the page, and not as shown.

Stimulation of the muscle. The muscle was stimulated by a single short discharge from a 0·03 μF. condenser, with the muscle shunted by a 1000 Ω. resistance. With a shock as brief as this the muscle may be assumed to be excited within 0·1 msec., and this delay time is considered as negligible. The condenser was charged and discharged by means of a Siemens high-speed relay. A second Siemens relay operated in series with the former by a hand key triggered the time base of the oscilloscope. The time interval between the moments of closure of the two relays was found to be less than 0·1 msec. The stimulating electrode consisted of a thin stainless steel wire stretched against the glass window in front of the photocell, the muscle being brought into contact with this window; or, in the 'directional' recording with the photomultiplier, the stimulating wire was held against the muscle with light pressure with a flexible support. The anodic connexion was made through the supporting stand. In both cases the point of stimulation lay in the centre of the illuminated patch of muscle. The muscle was not curarized.

Photocells. A single-stage vacuum cell (type VB 39: Cinema Television Ltd.) was used for the experiments with the simple optical arrangement. In the 'directional' experiments the light entering the photocell was of much lower intensity, and a 9-stage photomultiplier (RCA 931-A)

replaced the single-stage cell; it was run at 720 V., giving a 6000-fold amplification of the primary photocurrent.

The output from either type of photocell was passed through a series resistance, and the voltage across this resistance applied to the grid of the first amplifier valve. The values of the circuit components shown in Fig. 2. were determined by the requirements of a wide-frequency response combined with a low noise level.

Vibration of the building caused high-frequency mechanical interference which was sometimes greater than the electronic noise, but this was largely overcome by mounting the table on sponge rubber.

Electronic noise. The limiting factor in amplification was the electronic noise. There are two sources of noise: (*a*) the 'shot' noise caused by random fluctuations in the primary photocurrent; (*b*) the 'Johnson' noise from the resistance in series with the photocell. 'Shot' noise in the first valve is not important when using a high-gain amplifier. The use of a multiplier photocell effectively eliminates the 'Johnson' noise, because the 'shot' noise from the primary photocurrent greatly exceeds it after being amplified non-resistively in the photocell (Preisach, 1939). In a single-stage photocell the 'Johnson' noise preponderates over the 'shot' noise when the light intensity is below a certain level, and it is then that the multiplier photocell is superior. For this reason the photomultiplier was used in the 'directional' experiment when the light intensity was very low. Reduction of noise also requires that the width of the frequency band amplified should be as narrow as it is possible to make it without introducing distortion of the signal; and the light intensity should always be as high as possible. When using white light and 'non-directional' recording the noise was thus practically eliminated. On the other hand, when the transparency change was recorded by the 'directional' method, the earliest phase was not entirely free from noise. With monochromatic light the noise even with 'non-directional' recording was sometimes severe (see Fig. 7), but with 'directional' recording it overwhelmed the signal, and the latter combination could not be used.

Optimum thickness of muscle. The use of a thicker muscle would increase the modulation of the photocurrent, but this does not necessarily improve the signal-to-noise ratio. With an incident light of I_0 and an extinction coefficient of k, the intensity of the transmitted light is given by $I=I_0e^{-kd}$, where d is the length of the light path in the muscle. The noise can be taken as having an amplitude $A\sqrt{I}$ (where A is a constant), and the signal expressed as BId (where B is a constant). The signal-to-noise ratio is therefore $B/A\sqrt{(I_0)}de^{-\frac{1}{2}kd}$. This quantity has a maximum for a certain optimum value of d. Calculation shows that for frog's muscle, and directional recording in the line of the incident beam, this optimum is about 0·7 mm. for blue light, and 1·9 mm. for red light; it is consequently only about twice the thickness of a sartorius muscle. The function has a rather broad maximum, and there was little to be gained by using a double thickness of muscle.

Frequency response. (*a*) *Transparency.* When the single-stage photocell was used the capacity from the grid of the first valve to earth was made up of 'stray' capacity. The time constant was measured by injecting an impulse into the system, and recording its delay on the oscilloscope. The R.C. of the circuit for $R=1$ MΩ. (Fig. 2) proved to be 0·18 msec., which was small enough to give an undistorted record. In the case of the multiplier photocell with R at 100,000 Ω., shunted by a capacity of 0·001 μF., the calculated time constant was 0·1 msec. In both circuits the low-frequency response was determined by the coupling capacity (0·02 μF.) and the grid-leak resistance (100 MΩ.), the R.C. being 2 sec. The Mullard E800 oscilloscope itself has a low-frequency response corresponding to an R.C. value of 4 sec. (*b*) *Mechanical record.* The circuit used in conjunction with the crystal pick-up is shown in Fig. 2. The time constant of 100 msec. was sufficiently long to record the early relaxation with practically no distortion.

The delayed transparency change. A few experiments were made with d.c. amplification to record the transparency during the twitch and for some seconds afterwards. von Muralt (1934*a*, *b*) described a change produced by stimulation, and which decayed in a few minutes, but he did not use a high-frequency recording system and was unable to observe the onset of the change or the manner in which it followed on from the change which accompanies the twitch itself.

Rapid stretch. A curious transient change in transparency was seen in a muscle that was rapidly stretched. The stretch was applied by connecting the tibial end of the muscle with the moving arm of a Siemens high-speed relay, the movement being about 100 μ. and varied by adjusting the contact stops. The closing time of the unloaded relay is under 1 msec., but when loaded with a muscle under tension the delay is probably greater than this. A strip of rubber of approximately the same dimensions and opacity as the muscle was used as a control. The 'directional' recording system was employed. To damp out high-frequency oscillations which were caused by the sudden snap of the relay the muscle (or the rubber strip) was suspended with its surface in contact with a glass plate, the interface being kept moist with Ringer solution. Even when damped in this way the oscillations were fairly severe, as can be seen from the records (Fig. 9).

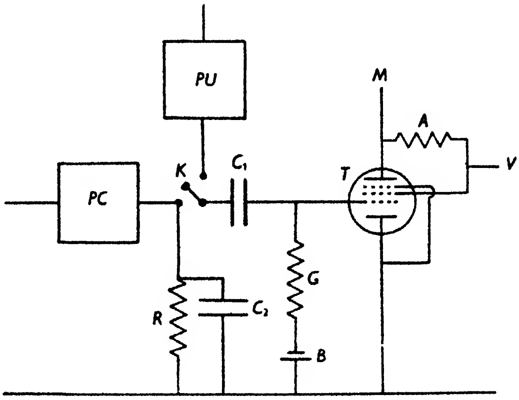


Fig. 2. Circuit for use with photocell, *PC*, or crystal pickup, *PU*. Coupling capacity, C_1, 0·02 μF. Grid leak resistance, *G*, 100 MΩ. Bias battery, *B*. Preamplifier valve, *T* (EF50, pentode): screen at voltage *V*, 250 V.; anode load, *A*, 20,000 Ω. Amplified voltage passes to Mullard oscilloscope, *M*. The key *K* selects either *PC* or *PU*. When the single stage photocell is used the resistance *R* is 1 MΩ., and the condenser C'_2 is removed. When the multiplier photocell is used *R* is 100,000 Ω., shunted with capacity C'_2, 0·001 μF.

RESULTS

Correlation of transparency with mechanical changes. To ensure the greatest possible freedom from noise the 'non-directional' optical system was used, with the single stage photocell, and white light. Fig. 3 shows photographs of the transparency change and of the early relaxation. To give a closer comparison seven such records were meaned and plotted together (Fig. 4). The optical and mechanical changes appear to start at the same time, within a fraction of a msec. Fig. 3 also shows the transparency change with lower amplification and slow time base; the initial change in the vertical direction can just be seen. The residue at the end of the twitch, showing as a positive displacement from the base line, is not a real change in transparency, but is an artefact due to the amplifier being condenser-coupled; the real residue is actually in the opposite direction.

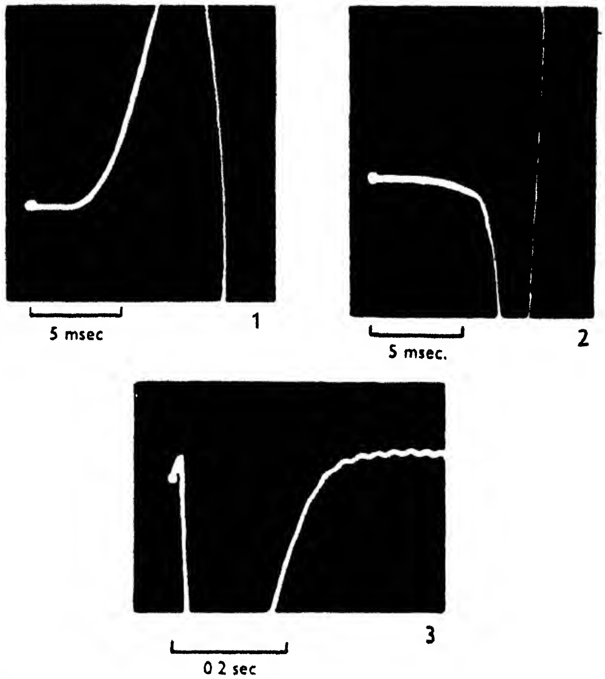


Fig. 3. 1, the opacity change with high amplification. 3, the same, but with low amplification. 'Non-directional' recording. The positive direction denotes an increase in photocurrent. 2, the mechanical record, showing the early relaxation.

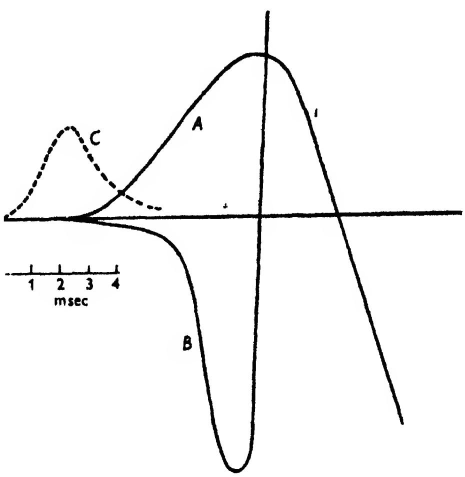


Fig. 4. Averaged records of opacity change, *A*; and of mechanical response, *B*. The time course of the action potential is also shown, *C* (not recorded, but as given by Katz, 1941).

The form and magnitude of the transparency change are unaltered when the stimulating electrode is removed out of the light beam. There can therefore be no possibility of the stimulating current itself being responsible for any part of the effect.

The action potential for the whole sartorius muscle is also shown in Fig. 4. (It was not recorded, but copied from Katz, 1941.) At present no particular significance can be attached to the fact that the peak of the action potential and the onset of the other events appear to be roughly coincident, for it has yet to be shown that this relation holds good under other conditions, e.g. at 0° C., and in any case the rising phase of the action potential of a single fibre (Kuffler, 1942) is probably more rapid than it is in the whole muscle.

Magnitude of the transparency change. If I denotes the resting photocurrent and ΔI the change on stimulation, the ratio $\Delta I/I$ is a measure of the effect. Two phases are considered, the early one during the early relaxation and the opposite phase which occurs later. With 'non-directional' recording the peak values are approximately:

	$\Delta I/I$ first peak	$\Delta I/I$ second peak
Violet light	1:1500	1:25
Red light	1:3000	1:50

The time course of the transparency change does not depend on the colour of the light.

Differentiation between absorption and scattering. The 'directional' method was used. The main transparency change, as seen with low amplification, is largely a scattering effect, and it reverses its sign as the telescope is swung from 0 to 90° (Fig. 5). The delayed transparency change, as described by von Muralt, also reverses; this is not clear from records taken with the relatively short time-constant amplifier, but the reversal is easily detected on the micro-ammeter which is in series with the output stage of the photomultiplier. Similarly, the effect of a small change of length is reversed as the telescope is moved from 0 to 90°.

On the other hand, the early optical change which starts with the early relaxation does *not* reverse in the same way (Fig. 5). At an angle of 0° to the incident beam it has the same sign as the much larger change which follows it; at 30° the secondary scattering effect is in the transition stage, and at 60° the two phases are in opposite directions (as they were with the 'non-directional' optical system). The effect at 90° is the same as at 60°.

The above observations lead to the conclusion that the early increase in transparency is essentially different from the later change. The latter, in view of its ready reversibility, must be caused by a change in the scattering power of the muscle, and its sign shows that it cannot be attributed to a localized thickening of the muscle at the electrode as the contraction commences, although it seems likely that changes in thickness of the muscle must to some extent affect

the transparency in the later stages. It is deduced also that the first effect cannot be due to the lengthening of the muscle during the early relaxation, as this should reverse with the angle; in any case, it can be calculated from Sandow's (1944) results that this lengthening should give less than one-thirtieth of the observed change at an angle of 0°. Consequently one might conclude that the

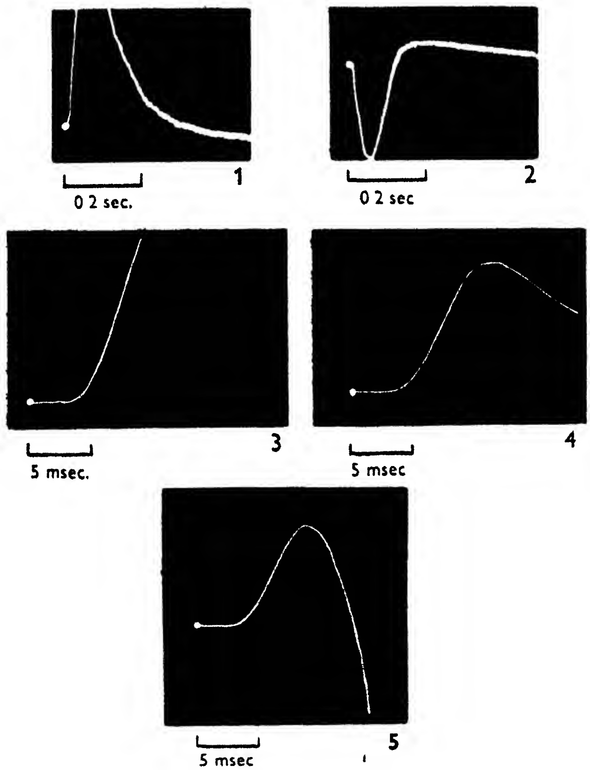


Fig. 5. The results with the 'directional' method. Records 1 and 2 show the response with low amplification, and with the telescope at 0° and 90° from the line of the incident beam. Records 3, 4 and 5 are with high amplification at 0, 30 and 60° from the line of the incident beam. The positive direction denotes an increase in photocurrent.

first phase of the optical change is caused by a decrease in *absorption*. Further evidence on this point was hoped for from an examination of the spectral distribution of the effect. The demonstration of absorption bands, or of a characteristic spectrum, would prove it to be an absorption phenomenon, as distinct from scattering, and might lead to the identification of the earliest reaction in stimulated muscle.

The spectral distribution of the first effect. At each wave-length the cathode-ray tube was photographed, the trace projected on squared paper and the

slope of the initial rise measured. The values so obtained were scaled to allow for variations in the light intensity at the different wave-lengths. The results of five experiments, with a total of 150 observations, are shown in Fig. 6. There does not appear to be a characteristic absorption spectrum, although it is possible there is a maximum around 450 mμ. The scatter in the results is largely caused by 'noise' which cannot be avoided when using monochromatic light.

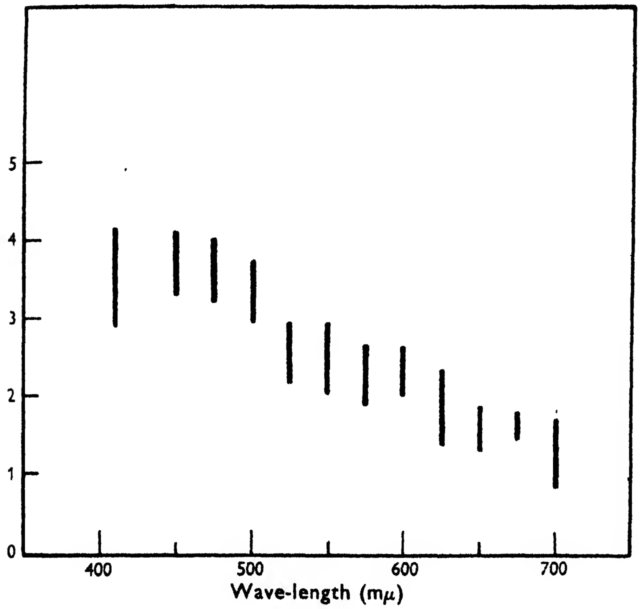


Fig. 6.

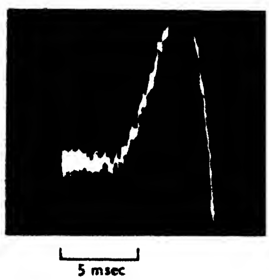


Fig. 7.

Fig. 6. The spectral distribution of the first transparency effect, showing the results of 150 observations distributed at wave-lengths from 400 to 700 mμ. At each wave-length the results are shown as a vertical line to represent the scatter, the length of each line being twice the average deviation from the mean. The vertical scale is in arbitrary units.

Fig. 7. A sample photographic record taken when using monochromatic light to show the 'noise'.

A sample record is shown in Fig. 7, and the importance of the 'noise' may be judged from this. It is proposed to make further observations using the intense mercury line at 365 mμ. as a source in the near ultraviolet, filtered from the visible radiation with a Wratten 18-A filter. This should help to decide whether there is truly a maximum at 450 mμ.

Polarized light. An attempt was made to locate the cause of the first transparency change in relation to the anisotropic and isotropic bands by working between crossed 'Polaroids'. To avoid overlap of the *A* and *I* bands of different fibres the sterno-cutaneous muscle was chosen for this purpose. This sheet of muscle is only one fibre thick. It was found that the early change in transparency was present, and of approximately the same magnitude as in

unpolarized light. The effect is therefore not confined to the isotropic bands. It is not yet possible to say whether the change is restricted to the anisotropic band, because the necessary *exact* comparison of the magnitudes of the signals with unpolarized light and with crossed polaroids has not yet been made; there is some difficulty in doing this owing to the great difference between the light intensities in the two cases.

The delayed transparency change. The transparency change recorded with a d.c. amplifier is shown in Fig. 8 for light received in the direction of the incident beam. It is seen that the change which remains after the twitch, and

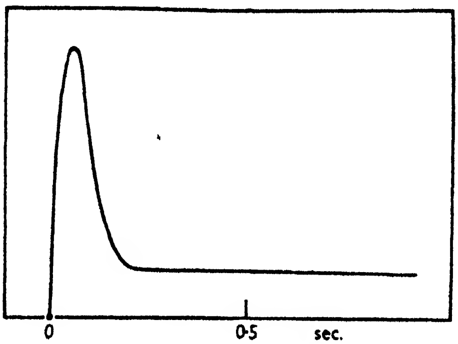


Fig. 8. The transparency change recorded with a d.c. amplifier, to show the delayed effect. 'Directional' recording in the line of the incident beam. The positive direction denotes an increase in photocurrent.

which decays in a matter of minutes as described by von Muralt, is in the same sense as the change during the twitch. Both phases are reversed in sign if the scattered light is measured.

Rapid stretch. The transparency changes caused by a rapid stretch of the muscle, and of the strip of rubber used as a control, are shown in Fig. 9. The effect with the rubber is attributable merely to a change in length. The muscle shows, in addition, a peculiar early transient effect. A muscle made inexcitable by soaking it in a potassium rich solution (20 times normal K) shows the same change. The effects following a rapid *release* of a previously stretched muscle are the reverse of these. By 'directional' recording it appears that the early transient effect is caused by an increase in the *scattering* of light, but this is not yet certain. An objection to the use of rubber for the control is that the viscosity or plasticity of the muscle has no counterpart in the rubber, for the latter behaves more like a simple spring. Perhaps the stretch effect in muscle can be reproduced in some other inert material, suitably chosen for its physical characteristics.

DISCUSSION

The early relaxation of a muscle is associated with a change in transparency of the muscle. The two events appear to start at precisely the same time. It seems fairly clear that the early transparency change has certain properties which are not shared by the change which occurs later, during the contraction proper. The latter phase is attributable largely to a change in the scattering power of the muscle, but there is evidence that the first phase, starting with the relaxa-

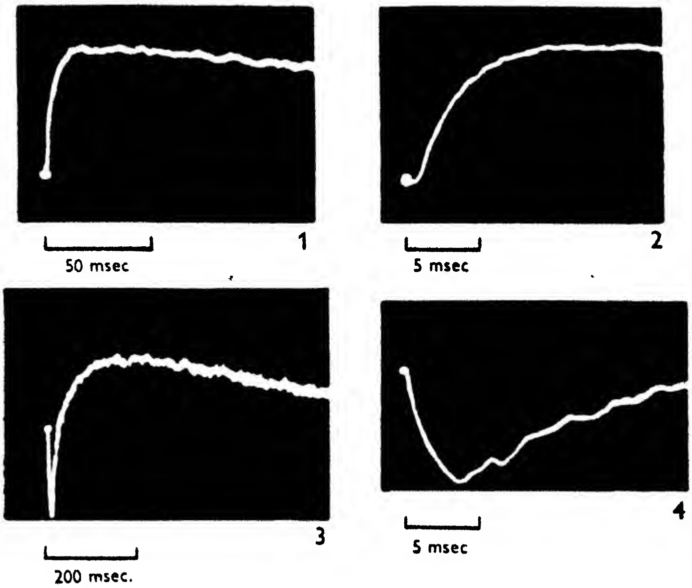


Fig. 9. Transparency changes as the result of a rapid stretch. 'Directional' recording at 50° from the line of the incident beam. The vertical direction denotes an increase in photocurrent. 1 and 2 show the effect with the 'control' rubber strip; 3 and 4 are for muscle, and show the early rapid transient change which is absent in the 'control'.

tion, may be caused by a decrease in the true absorbing power of the muscle substance. A determination of the spectral distribution has not confirmed this supposition, and the question is still undecided. Further experiments may show that in spite of the evidence to the contrary the first phase is really due to scattering. It may prove that the difference between the two phases is connected with some subtle dissimilarity in the mechanisms by which the light is scattered in the two cases. The many units which compose muscle—the molecules, the 'micelles', the fibrils, the fibres—may all play some part in scattering the light. Moreover, the scattering power of a particular type of unit may be determined not only by its chemical constitution or environment, but also by its degree of organization; the more complete the orientation of the particles among themselves the smaller will be their scattering power. Again, such

processes as the gelation of a colloidal solution, associated with changes in particle size, may give rise to changes in opacity (Krishnamurti, 1929).

The possibility has not been ignored of the change being due to an alteration in the reflecting power of the muscle membrane. To test this it will be necessary to record changes in intensity of light scattered and reflected backwards towards the source of light. The ideal way of distinguishing between absorption on the one hand, and scattering and reflectivity on the other, would be to record the complete three-dimensional 'polar diagram' of the effect.

SUMMARY

1. A study has been made of the changes in transparency of frog's skeletal muscle during a twitch. The source of light was a tungsten filament lamp combined, in some of the experiments, with monochromatic colour filters.

2. The mechanical response, starting as a transient relaxation, was recorded under the same conditions by means of a crystal pick-up. The transparency change and the early relaxation appear to commence at exactly the same instant, within a fraction of a msec., i.e. at about 2 msec. at 15° C.

3. An attempt was made to distinguish between *scattering* and *absorption* of the light. It is possible that the first phase, during the early relaxation, is an absorption phenomenon, in contrast with the later change which is largely a scattering effect. An examination of the spectral distribution of the first phase has not revealed any characteristic absorption bands.

4. Experiments were made to test whether a rapid stretch of the muscle gave rise to any transparency change other than that due to a change of thickness. The muscle was stretched about 100 μ. A strip of rubber was used as the control. An early rapid transient effect was observed, but it has not yet been investigated in detail.

I wish to express my thanks to the Rockefeller Foundation for a grant which made this work possible.

REFERENCES

Buchthal, F., Knappeis, G. G. & Sjöstrand, T. (1939). *Skand. Arch. Physiol.* **82**, 225.
Hill, A. V. (1948). *J. Physiol.* **107**, 29 *P*.
Katz, B. (1941). *J. Neurophysiol.* **4**, 207.
Krishnamurti, K. (1929). *Proc. Roy. Soc.* A, **122**, 76.
Kuffler, S. W. (1942). *J. Neurophysiol.* **5**, 309.
von Muralt, A. (1934*a*). *Pflüg. Arch. ges. Physiol.* **234**, 233.
von Muralt, A. (1934*b*). *Pflüg. Arch. ges. Physiol.* **234**, 653.
Preisach, F. (1939). *Wireless Engr.*, **16**, 169.
Sandow, A. (1944). *J. cell. comp. Physiol.* **24**, 221.
Schaefer, H. & Göpfert, H. (1937). *Pflüg. Arch. ges. Physiol.* **238**, 684.
Urban, F. & Peugnet, H. B. (1938). *Proc. Roy. Soc.* B, **125**, 93.

J. Physiol. (1949) 108, 303–314 612.391:612.014.461.3

EFFECTS OF DEHYDRATION ON ADULT AND NEWBORN RATS

By H. HELLER

From the Department of Pharmacology, University of Bristol

(*Received* 10 *June* 1948)

There is some indication that decreases of the body water load fail to induce a substantial rise of the urinary osmotic pressure in very young children: Young & McCance (1942) investigating infants aged 5–12 weeks who suffered from gastroenteritis, were able to show that the osmotic pressure of the urine rarely rose to that of urine samples obtained from adults at comparable minute volumes. This apparent inability to concentrate the urine under stress was also seen in a normal full-term infant of less than 48 hr., who secreted a hypotonic urine even when its fluid intake was restricted by the omission of two feeds (McCance & Young, 1941). It was shown by Heller (1944) that the osmotic pressure of urine samples collected from normal infants during the first 2 days after birth, i.e. when fluid intake is low, did not reach the figures obtained in urines of normal adults. However, in the absence of any quantitative criterion for the degree of dehydration in these infants, it might be asked whether the conditions were stringent enough (the watery nature of the food should here be considered) to exclude a rise of urinary concentration after further deprivation of water. Since the limitations of clinical tests prevent deliberate withdrawal of fluid from normal newborn infants for more than a short period, it was decided to compare the effects of dehydration in newborn rats deprived of fluid for 24 hr. with those in adult rats suffering from approximately the same relative extrarenal water loss.

METHODS

Experimental animals. Male albino rats, 12–48 hr. old, and male adult rats (weighing 180–310 g.) were used. The adults, which were of the same strain as the newborn animals, were kept on a standard diet containing 18% casein. The newborn rats were removed from their cage after they had suckled at least once and were then kept at 33/34° C. for 24 hr. by placing them in a thermoregulated oven. As milk supplies both food and water to the newborn animals, the adult rats were deprived of both water and food during the period of dehydration.

Determination of extrarenal water loss. The extrarenal water loss of newborn rats was determined by weighing the animals on an analytical balance. Animals which passed faeces or urine were

discarded. Adult rats were put singly into glass metabolism cages fitted with urine/faeces separators. The air temperature in these as well as in the experiments on newborn rats was thermostatically controlled.

Collection of urine. The bladder of the newborn animals was emptied at the beginning of the experiment by applying suprapubic pressure. At the end of the period of dehydration the animals were killed by decapitation and the urine withdrawn by bladder puncture. The quantity of urine thus obtained was measured by delivering it into a tared weighing bottle. From adult rats spontaneously voided urine was collected under paraffin oil; prodding and, if necessary, application of suprapubic pressure ensured complete emptying of the bladder at the end of the experimental period.

Collection of blood. The newborn animals were decapitated, the blood mixed with heparin on a porcelain plate and quickly drawn into capillary tubes of about 1 mm. internal diameter. The tubes were centrifuged and the corpuscles removed by cutting off the appropriate part of the capillary. The plasma was then blown into small weighing bottles. The adult animals were lightly anaesthetized with ether and the blood obtained from the severed vessels of the neck. Heparin plasma was used throughout.

Determination of specific gravity of urine. Heller's (1941) apparatus was used.

Freezing-point determinations. A Beckmann apparatus for small quantities of fluid was used. Values for Δ were converted into milliosmols by the usual formula: C (in m.osmol.) $=\Delta/1{\cdot}86 \times 1000$.

Determinations of water content. Samples of whole blood, plasma and urine were dried at 105° C. for 48 hr. Muscle samples (the recti abdominis were used in both adult and newborn rats) were dried at 105° C. and the fat extracted according to the procedure of Eichelberger (1941).

Analytical methods. Chloride in plasma was determined by Whitehorn's (1921) method, and in urine by that of Volhard (1878). Sodium in plasma and urine was determined by the method of McCance & Shipp (1931); potassium in plasma and urine by Kramer & Tisdall's method as modified by McCance & Shipp (1933). Urea in plasma and urine was determined according to Lee & Widdowson (1937).

RESULTS

Extrarenal water loss. It has been shown in a previous paper (Heller, 1947*b*) that during short-term experiments (3 hr.) the extrarenal water loss of newborn rats kept at 30/31° C. was of similar magnitude to that of adult animals kept at 20/21° C. When the period of dehydration was extended to 24 hr. it was found that the extrarenal water loss of newborn rats kept at 30/31° C. was somewhat smaller ($4{\cdot}0 \pm 0{\cdot}09$ g./100 g./24 hr. (S.E. of mean of seventeen observations)) than that of adults ($5{\cdot}5 \pm 0{\cdot}20$ (27) g./100 g./24 hr., $t=6{\cdot}84$, $P<0{\cdot}001$). Raising of the environmental temperature of the newborn animals to 33/34° C. led to extrarenal losses ($5{\cdot}3 \pm 0{\cdot}28$ (20) g./100 g./24 hr.) which compared well with those of adults at 20/21° C. ($t=1{\cdot}20$, $P>0{\cdot}2$). The investigations on newborn rats described subsequently were therefore made at 33/34° C.

Specific gravity of the urine. In adult rats specific gravities over 1·060 were observed in individual animals, and determinations of the freezing-point depression of urine samples from dehydrated adult rats gave the following typical figures in a random series: 2·13, 2·08, 1·68, 1·81 and 2·42 osmol./l. The average maximal capacity of the adult human kidney to concentrate solutes is 1·4 osmol./l. (Gamble, 1947). These results agree with those of Adolph (1943), who reports the maximal urinary concentrations of chloride as 0·60 osmol. in the rat and 0·37 osmol. in man.

Newborn rats. Table 1 shows that the mean specific gravity of urine samples from dehydrated newborn rats did not differ significantly from that of urine samples from normal animals. However, it was not always possible to empty the bladder of newborn rats completely by applying suprapubic pressure. Any residual urine at the beginning of the experiment was thus included in the amount obtained by bladder puncture at the end of the period of dehydration.

TABLE 1. Effect of withdrawing fluid and food for 24 hr. from adult and newborn rats on the specific gravity of the urine

Age and environmental temperature	Specific gravity of urine		't'
	Normal rats	Dehydrated rats	
Adult rats at 20/21° C.	1·017 ± 0·0016 (20)	1·056 ± 0·0022 (20)	—
Newborn rats at 20/21° C.	1·011 ± 0·0005 (10)	1·012 ± 0·0005 (21)	$t = 0{\cdot}084$ $P > 0{\cdot}9$
Newborn rats at 33/34° C.:			
Series A*	1·015 ± 0·0005 (11)	1·016 ± 0·0006 (17)	$t = 0{\cdot}777$ $P > 0{\cdot}4$
Series B*	1·013 ± 0·0005 (14)	1·014 ± 0·0004 (18)	$t = 0{\cdot}840$ $P > 0{\cdot}4$

* The animals of the two series were of the same strain, but the mothers received a different stock diet.
Results are given as means with their standard errors.
Number of animals in brackets.

Admixture of urine of low specific gravity may thus have 'masked' an increase of specific gravity due to dehydration. The mean amount of residual urine in a series of fourteen normal newborn rats was therefore determined. The animals were made to deliver as much urine as possible by suprapubic pressure, were then decapitated and the residual urine obtained by puncture of the bladder. It amounted to 0·45 ± 0·084 g./100 g. rat and had the mean specific gravity of 1·013 ± 0·0005. The mean amount of urine obtained in 24 hr. from a series of eighteen litter mates kept at 33/34° C. was 2·99 ± 0·344 g./100 g. and had the specific gravity of 1·014 ± 0·0004. On the assumption that the mean amount of residual urine was much the same in the dehydrated as in the normal litter mates the true specific gravity of the urine secreted during the 24 hr. period of dehydration could be determined from the following expression:

$$S_X = \frac{(W_{24} \times S_{24}) - (W_0 \times S_0)}{W_{24} - W_0},$$

where W_{24} = weight of urine collected after the period of dehydration.
W_0 = weight of residual urine.
S_{24} = specific gravity of urine collected after the period of dehydration.
S_0 = specific gravity of residual urine.

S_X for the example given was 1·0142, i.e. the true value of the mean specific gravity of the urine collected during the period of dehydration did not differ significantly from the mean specific gravity of the actual samples which contained about 15% (W/W) of residual urine.

Concentration and renal excretion of plasma crystalloids. Table 2 shows the effect of withdrawing fluid and food for 24 hr. on the concentration of chloride, sodium, potassium and urea in the plasma and the urine of adult and newborn rats. It should be pointed out that the concentration of these crystalloids in the body fluids is stated in terms of m.equiv./l. water and not as the amount of crystalloid per unit volume of the body fluid. It is the former which represents their osmotically effective concentration thus permitting the comparison of different body fluids. To calculate concentrations per unit volume of water, the solids in plasma and urine had to be estimated. The following figures were obtained (means and their standard errors): plasma solids of normal adult rats $= 8{\cdot}2 \pm 0{\cdot}17$ (12) g./100 g., plasma solids of dehydrated adult rats $= 8{\cdot}4 \pm 0{\cdot}20$ (12) g./100 g., plasma solids of normal newborn rats $= 4{\cdot}9 \pm 0{\cdot}22$ (21) g./100 g., plasma solids of dehydrated newborn rats $= 6{\cdot}1 \pm 0{\cdot}24$ (20) g./100 g. Urinary solids were calculated from determinations of specific gravity. Long's coefficient (2·6) and Haeser's coefficient (2·33) proved unsatisfactory. The specific gravity (at 21° C.) and the amount of solids in thirty-seven urine samples from adult and newborn rats were therefore determined and a coefficient fitted to the results. The most satisfactory value for the coefficient was 1·50. The mean urinary specific gravities recorded in Table 1 were used with this coefficient.

Differences between the plasma chemistry of normal adult and normal newborn rats will be noted from Table 2; the mean plasma concentration of chloride in the newborn rats was significantly lower ($t = 6{\cdot}38$, $P < 0{\cdot}001$) than that in adults, the concentrations of potassium and urea were significantly higher ($t = 3{\cdot}76$, $P < 0{\cdot}001$; $t = 3{\cdot}82$, $P < 0{\cdot}001$), that of sodium was much the same ($t = 1{\cdot}42$, $P > 0{\cdot}1$). The variations in the plasma electrolyte concentrations of individual animals were too great (see values for S.E. in Table 2) to permit reliable conclusions as to minor changes after the deprivation of fluid for 24 hr. However, the plasma urea of the dehydrated newborn animals had undoubtedly much increased ($t = 3{\cdot}51$, $P < 0{\cdot}001$). In comparison, there was no significant difference ($t = 1{\cdot}65$, $P < 0{\cdot}1$) between the mean values for plasma urea in normal and in dehydrated adult rats.

The figures for the urine/plasma ($= U/P$) ratio of the four substances analysed show (Table 2) that adult rats respond to withdrawal of fluid for 24 hr. with a 4- to 9-fold increase of the U/P ratios. Newborn animals which had been deprived of fluid for the same period and which had suffered from approximately the same extrarenal loss of water, concentrated the four crystalloids to a much lower extent: the U/P ratios of chloride and potassium changed but little, that of sodium increased much less than in the adults, and that of urea decreased in spite of the fact that the mean plasma urea concentration had risen by about 45%.

The estimations of the main osmotically active constituents of the plasma and urine in the newborn rats made it possible to obtain an indication of the

TABLE 2. Effect of withdrawing fluid and food for 24 hr. from adult and newborn rats

	Normal animals: Plasma ($=P_1$) (m.equiv./l. water)	Normal animals: Urine ($=U_1$) (m.equiv./l. water)	Dehydrated animals: Plasma ($=P_2$) (m.equiv./l. water)	Dehydrated animals: Urine ($=U_2$) (m.equiv./l. water)	Normal animals (U_1/P_1)	Dehydrated animals (U_2/P_2)	Concentration factor*
Adult rats							
Chloride	91·5 ± 1·32 (12)	15·2 ± 2·50 (11)	88·1 ± 1·70 (12)	84·9 ± 5·49 (12)	0·17	0·96	5·7
Sodium	144·3 ± 3·10 (12)	15·1 ± 3·13 (11)	151·7 ± 1·39 (12)	62·5 ± 2·95 (12)	0·10	0·41	4·1
Potassium	5·8 ± 0·10 (12)	31·0 ± 4·39 (11)	6·0 ± 0·12 (12)	282·9 ± 19·96 (12)	5·3	47·2	8·9
Urea	7·6 ± 0·39 (12)	211 ± 38·9 (12)	6·7 ± 0·45 (12)	913 ± 76·3 (12)	27·8	136·3	4·9
Newborn rats							
Chloride	69·9 ± 3·13 (31)	32·0 ± 5·66 (36)	69·9 ± 2·68 (28)	28·3 ± 5·50 (32)	0·46	0·41	0·9
Sodium	131·6 ± 8·36 (33)	20·9 ± 1·48 (31)	113·4 ± 5·46 (33)	30·3 ± 5·44 (23)	0·16	0·27	1·7
Potassium	10·8 ± 1·33 (33)	59·8 ± 9·73 (40)	8·5 ± 0·54 (32)	57·8 ± 8·04 (32)	5·5	6·8	1·2
Urea	11·1 ± 0·83 (34)	67·2 ± 6·54 (34)	16·2 ± 1·19 (32)	65·2 ± 5·61 (32)	6·0	4·0	0·7

* Concentration factor = ratio U_2/P_2 divided by ratio U_1/P_1.
Results are given as means with their standard errors.
Number of animals in brackets.
Adult rats kept at 20/21° C., newborn rats kept at 33/34° C.

TABLE 5. Effect of withdrawing fluids and food for 24 hr. from adult and newborn rats

	Adult rats: Normal animals	Adult rats: Dehydrated animals	Adult rats: 't'	Newborn rats: Normal animals	Newborn rats: Dehydrated animals	Newborn rats: 't'
Whole blood solids (g./100 g.)	20·5 ± 0·29 (12)	21·1 ± 0·39 (12)	$t=1·25$ $P>0·2$	14·1 ± 0·39 (34)	15·4 ± 0·32 (34)	$t=2·55$ $P<0·02$
Plasma solids (g./100 g.)	8·2 ± 0·17 (12)	8·4 ± 0·20 (12)	$t=0·08$ $P>0·9$	4·9 ± 0·22 (21)	6·1 ± 0·24 (20)	$t=3·55$ $P<0·01$
Haematocrit	49·7 ± 0·91 (12)	51·9 ± 1·00 (12)	$t=1·63$ $P>0·1$	34·3 ± 0·79 (22)	39·4 ± 1·38 (22)	$t=3·21$ $P<0·01$
Red cell count (in 1000)	8558 ± 111 (12)	8612 ± 94 (12)	$t=0·04$ $P>0·9$	3347 ± 53 (17)	3579 ± 110 (12)	$t=2·11$ $P<0·05$
Muscle solids (g./100 g. fat-free muscle)	24·6 ± 0·23 (12)	24·3 ± 0·22 (12)	$t=0·95$ $P>0·3$	12·1 ± 0·36 (19)	13·1 ± 0·34 (20)	$t=2·04$ $P<0·05$

Results are given as means with their standard errors.
Number of animals in brackets.
Adult rats kept at 20/21° C.; newborn rats kept at 33/34° C.

osmolar concentrations of these body fluids. The following formula (McCance & Young, 1941), which expresses the tonicity of the urine, was used:

$$\frac{(\text{m.equiv. of } Cl^- \text{ in urine} \times 2) + \text{m.equiv. of urea}}{(\text{m.equiv. of } Na^+ \text{ in plasma} \times 2) + \text{m.equiv. of urea}} = \frac{U_c}{P_c}.$$

Table 3 shows that the ratio U_c/P_c in the adult rats increased more than four times during the period of dehydration; in other words, the urine of these animals was strongly hypertonic. A comparison with the U_c/P_c ratios of the newborn rats shows that there was no significant difference between the

TABLE 3. Effect of withdrawing fluids and food for 24 hr. from adult and newborn rats on the osmolar concentration of plasma and urine

	Plasma, osmolar concentration ($=P_c$) (m.osmol./l.)	Urine, osmolar concentration ($=U_c$) (m.osmol./l.)	U_c/P_c
	Adult rats		
Normal animals	275 ± 2·8	222 ± 40·1	0·80 ± 0·138
Dehydrated animals	278 ± 4·9	992 ± 73·0	3·65 ± 0·249
	Newborn rats		
Normal animals	279 ± 13·1	180 ± 14·6	0·67 ± 0·092
Dehydrated animals	248 ± 13·8	132 ± 10·5	0·55 ± 0·044

Results are given as means with their standard errors.
Adult rats kept at 20/21° C., newborn rats kept at 33/34° C.

dehydrated and the normal newborn animals ($t=1·19$, $P>0·3$), i.e. the urine remained hypotonic. Correction for residual urine was unnecessary in this instance as the osmolar concentration of the urine of the dehydrated animals was lower than that of the urine of the normal series. This difference may not have been fortuitous but may have been due to the decreased excretion of electrolytes (see Table 4) by the dehydrated rats.

Urine volume. The evidence so far presented shows that adult rats under the stimulus of a withdrawal of fluid for 24 hr. controlled the loss of body water by elaborating a highly hypertonic urine but that newborn rats were apparently unable to regulate their urine concentration by the same renal mechanism. It seemed likely, therefore, that dehydrated newborn rats would excrete a larger volume of urine than adult animals suffering from the same degree of extrarenal water loss. The mean volume of urine of a series of twenty dehydrated newborn rats was 3·3 ± 0·25 c.c./100 g./24 hr. (The urine volumes of individual newborn animals were corrected by deducting the values for residual urine obtained from paired litter controls.) The mean volume of urine of a series of twenty-seven adult rats which were deprived of water and food for the same period was 1·5 ± 0·13 c.c./100 g./24 hr. The difference is significant ($t=6·38$, $P<0·001$). The mean extrarenal loss of water was the same in both series ($t=0·58$, $P>0·8$).

The mean urine volume per 24 hr. of normally hydrated newborn rats as estimated from the hourly output of 14 animals kept at 30/31° C. for 5 hr. (Heller, 1947*b*) was 6·8 ± 0·59 c.c./100 g./24 hr. The dehydrated newborn rats excreted 3·3 ± 0·25 c.c./100 g./24 hr., i.e. their urine volume had decreased to about half of the normal mean. The urine volume of four adult rats with food and water *ad lib.* measured on three consecutive days was 9·3 ± 1·06 c.c./100 g./24 hr. The mean urine volume of the dehydrated adult rats was 1·5 ± 0·13 c.c./100 g./24 hr., i.e. it was reduced to about 15% of the normal.

The amounts of chloride, sodium, potassium and urea excreted by adult and newborn rats in 24 hr. (Table 4) were computed from the preceding figures for mean urine volumes and from the data used for Table 2. The difference between the output of potassium of the dehydrated newborn and of the dehydrated adult animals will be noted.

TABLE 4. Effect of withdrawing fluids and food for 24 hr. from adult and newborn rats on the amounts of crystalloids excreted in the urine

	Amounts excreted in urine in 24 hr. (m.equiv./100 g. body weight/24 hr.)			
	Chloride	Sodium	Potassium	Urea
	Adult rats			
Normal animals	0·142	0·140	0·288	1·960
Dehydrated animals	0·116	0·086	0·388	1·254
Ratio: Dehydrated animals / Normal animals	0·82	0·61	1·35	0·64
	Newborn rats			
Normal animals	0·218	0·142	0·407	0·457
Dehydrated animals	0·091	0·097	0·187	0·210
Ratio: Dehydrated animals / Normal animals	0·42	0·68	0·46	0·46

Adult rats kept at 20/21° C., newborn rats kept at 33/34° C.

Water content of blood and skeletal muscle. Table 5 shows that deprivation of fluid for 24 hr. upsets the internal environment of newborn rats to a higher degree than that of adult animals. The concentration of water in the blood of the adult rats (as judged from estimations of the solids in whole blood and plasma) remained much the same after the withdrawal of fluid for 24 hr., but the same measure produced a significant rise of the blood solids and plasma solids in newborn animals. Determinations of red cell volume and red cell counts agreed fully with these results. The values obtained in adult animals showed little change, but significant increases were observed in the newborn rats. Withdrawal of fluid for 24 hr. thus led to pronounced haemoconcentration (and therefore inferentially to a decrease of the blood volume) in the newborn rats but not in the adults.

To check the normal decrease of body water in growing rats, litters of newborn rats were divided into two groups. One group was killed immediately,

and blood solids, muscle solids and red cell volume were estimated; the other group was left with the mother for a further 24 hr. The same determinations were then made, but no significant differences were found between the data obtained from the two series. The body weight of the second group of animals had increased, showing that suckling had proceeded in a normal manner. These results conform with those of Bruner, van de Erve & Carlson (1938), who failed to find significant differences in the number and the volume of the red blood corpuscles of suckling rats when determining these values from day to day during the first days after birth.

It will be seen from Table 5 that the skeletal muscle of newborn rats deprived of fluid for 24 hr. lost more water than that of the adult animals. The difference between the mean amounts of water in the muscle samples from normal and dehydrated *adult* rats was in fact not statistically significant. This result is somewhat puzzling in view of the marked loss of body water by the latter animals. Did water released by metabolic changes cover the deficit? This may be part of the explanation. However, it may well be that the maintenance of the water content of the muscle in the adult rat after 24 hr. of dehydration was due to a 'preferential' water loss from other tissues. De Boer (1946) showed, for instance, that in dogs in acute and chronic dehydration much less water was lost from the muscles than from the skin.

DISCUSSION

The results of the experiment on adult rats show that these animals responded to withdrawal of water (and food) for 24 hr. in much the same manner as human beings (Nadal, Pedersen & Maddock, 1941) and dogs (Elkington & Taffel, 1942; Elkington & Winkler, 1944). The ability of the adult rat kidney to perform osmotic work proved to be greater than that of the other two species mentioned: urines with specific gravities over 1·060 and osmolar concentrations over 2·0 osmol./l. were secreted by dehydrated adult animals. The mean urine volume during the period of dehydration fell from 9·3 to 1·5 c.c./100 g./24 hr. As one would expect in a small species with a relatively large body surface extrarenal water loss was high ($5{\cdot}5 \pm 0{\cdot}20$ g./100 g./24 hr.). The obligatory expenditure of water (made up of the amounts lost from skin and lungs and the volume needed for the adequate excretion of solutes in the urine) must clearly have made demands on the extracellular fluid. However, not only was the normal ionic composition of the plasma on the whole maintained, but the extracellular fluid volume and particularly the plasma volume were also well defended: no marked increase in plasma solids and no change in the haematocrit and the red cell count were found after 24 hr. of dehydration. In view of the magnitude of the total water loss, the avoidance of haemoconcentration could only have been achieved by a shift of intracellular water. The increase in excretion of the intracellular electrolyte potassium agreed with this assumption.

The plasma potassium level of the dehydrated rats was not raised. Similarly, a decrease of cell potassium without an increase in plasma potassium has recently been demonstrated by Elkington, Winkler & Danowski (1948) in adult dog deprived of water.

The high efficiency of the kidneys of dehydrated adult rats in performing osmotic work or, in other words, in increasing tubular water reabsorption, has been demonstrated. Estimations of glomerular function during the period of dehydration were beyond the scope of this investigation. However, it seems likely that reduction of the glomerular filtration rate is not an important response of the adult rat to losses of water of the degree described. Dicker & Heller (1945) found little decrease of inulin clearance in adult rats at minute volumes of urine as low as 0·0035 c.c./100 g. Moreover, no significant rise of the plasma urea, such as would occur with a substantial decrease of the glomerular filtration rate (Gamble, 1947), could be shown in the adult animals.

How do newborn rats, deprived of fluid and food for 24 hr., differ in their response from that of adult animals? The data presented show that the specific gravity of the urine hardly increased. Estimations of the urine/plasma ratios of chloride, sodium, potassium and urea in the dehydrated newborn animals agreed with this finding. More water per unit weight of urinary solids was therefore excreted by the dehydrated newborn rats: newborn rats, in which the extrarenal loss during the period of dehydration was so adjusted as to be much the same as that of the adults, excreted 3·3 c.c. urine/100 g./24 hr.; adult animals excreted 1·5 c.c./100 g./24 hr. The difference, though significant in that particular series, is surprisingly small. It suggests the possibility that newborn rats when deprived of fluid regulate renal loss of water by a process different from that in adult rats, where expenditure of water in the urine is mainly reduced by an increase of renal osmotic work, i.e. by invoking a tubular mechanism. A comparison between the mean urine volumes of the normal and the dehydrated newborn rats indicated a decrease during the 24 hr. of dehydration. Withdrawal of fluid from the newborn animals led to little increase of osmotic work by the tubules. The fall in urine volume may therefore have been due to a decrease of the glomerular filtration rate. Such a response to a decrease in body water is well known in human adults and adult dogs suffering from excessive dehydration, i.e. from a degree of water loss which produces haemoconcentration and a decrease of the blood volume (Smith, 1937). A substantial decrease of the rate of glomerular filtration in the dehydrated newborn rat is also suggested by the significant rise of plasma urea. It has been shown by Gamble (1947) that 'the concentration of urea in plasma is the concentration required in the glomerular filtrate for removal of the daily urea load. With reduction of filtration capacity there is proportionate increase of urea concentration in plasma and filtrate which permits successful removal of the load.' Plasma urea in man is not found appreciably above the 'normal

range' until the glomerular filtration rate has been reduced to less than half. The ratio of amounts of urea excreted by normal animals to amounts of urea excreted by dehydrated animals was comparable in adult and newborn rats, and this agrees with Gamble's concept. The amount of sodium excreted in 24 hr. was much the same in the dehydrated newborn and adult animals, the excretion of chloride was somewhat less in the dehydrated newborn rats. The difference between the amounts of potassium excreted by newborn rats and adults during the period of deprivation was, however, striking. While dehydrated adult rats excreted *more* potassium in the urine than well-hydrated animals, renal elimination of potassium by the dehydrated newborn animals fell by about half. No evidence for a rise in plasma potassium in the newborn rats could be obtained. Any potassium released by the breakdown of tissue constituents (the metabolic rate of newborn rats at the environmental temperature chosen approached that of adult rats at room temperature: Gullick, 1937; Herrington, 1940) seems, therefore, to have been retained in the cells. The same phenomenon was noticed by Elkington & Winkler (1944) in dehydrated adult dogs when kidney function was experimentally impaired.

It would thus appear that two factors combine in reducing the extracellular fluid volume of dehydrated newborn rats: (1) the obligatory expenditure of water (renal and extrarenal), and probably (2) loss of water to the intracellular fluid phase due to the osmotic action of increasing amounts of ionized potassium in the cells. Evidence indicating a decrease of the extracellular fluid phase has been presented. In contrast to adult rats suffering from a comparable water loss, the plasma solids of the dehydrated rats were found to be significantly increased, as were the haematocrit values and the red cell count. These findings show that the defence of newborn rats against dehydration was less effective than that of adult animals under comparable conditions of stress, i.e. the internal environment of the adults deviated less from the normal.

Why do dehydrated newborn rats fail to excrete a highly hypertonic urine or, in other words, why do the renal tubules of the newborn rat fail to respond to a decrease of the body water load by increasing their osmotic work? There is some evidence suggesting that the neurohypophysial mechanism, which regulates urinary concentration in the adult, is at fault. An inadequacy of this mechanism may be caused by insufficient production of the antidiuretic hormone (as in diabetes insipidus), by a lack of response of the renal tubules to the antidiuretic hormone (as in some patients with chronic nephritis), by the insufficient development of or interference with integrative structures (e.g. the osmoreceptors) or by some combination of these factors. The pituitary glands of newborn rats have been shown to contain only about one-tenth of the antidiuretic principle found in adults when calculated per 100 g. body weight and even less when calculated per mg. dry gland tissue (Heller, 1947*a*). A hypofunction of the pars nervosa may therefore be one of the factors

concerned in this species. The sensitivity of the renal tubules of newborn rats to the antidiuretic hormone has not been examined. However, it has been shown that the renal tubules of newborn infants are relatively insensitive to posterior pituitary extracts (Heller, 1944), and that the renal tubular apparatus is less mature in newborn rats than in newborn children (Baxter & Yoffey, 1948). The developmental state of the hypothalamic structures concerned with neurohypophysial secretion does so far not seem to have been investigated in the newborn.

SUMMARY

1. The effects of withdrawing water and food for 24 hr. were investigated in newborn rats kept at 33/34° C. and adult rats kept at 20/21° C. The mean extrarenal water loss at these environmental temperatures was approximately the same in the two series.

2. The mean specific gravity of the urine of the adult animals during the period of dehydration rose to $1{\cdot}056 \pm 0{\cdot}002$ and osmolar concentrations over 2·0 osmol./l. urine were encountered. The kidney of the adult rat is thus able to concentrate urine more highly than that of adult man. In contrast, the mean specific gravity of the urine of the newborn rats did not increase significantly on dehydration.

3. The mean urine/plasma ratio of chloride, sodium, potassium and urea in the dehydrated adult rats increased 4- to 9-fold. Their plasma urea remained normal. The mean urine/plasma ratios of chloride, sodium and potassium in the newborn rats altered little during the period of dehydration; the U/P ratio of urea decreased even though the mean plasma urea had risen by about 45%.

4. The mean urine volume of adult rats fell from $9{\cdot}3 \pm 1{\cdot}06$ c.c./100 g./24 hr. to $1{\cdot}5 \pm 0{\cdot}13$ c.c./100 g./24 hr. and that of newborn rats from $6{\cdot}8 \pm 0{\cdot}59$ c.c./100 g./24 hr. to $3{\cdot}3 \pm 0{\cdot}25$ c.c./100 g./24 hr.

5. The renal output of potassium in adult rats increased during the period of dehydration. However, dehydrated newborn rats excreted less potassium than normal newborn animals.

6. Withdrawal of fluid and food from adult rats for 24 hr. failed to produce significant changes of the water content of the plasma and skeletal muscle, the haematocrit and the red cell count. In contrast, the water content of the plasma of newborn rats decreased significantly, and the haematocrit and the red cell count showed marked increases; significant amounts of muscle water were lost by the dehydrated newborn animals.

7. It was concluded from these results that adult rats, after the moderate losses of body water incurred during 24 hr. of dehydration, maintained their extracellular fluid volume by increasing tubular water reabsorption, and by a shift of water from the intracellular fluid space. No evidence for a substantial increase of tubular water reabsorption was obtained in dehydrated newborn

rats suffering from a similar degree of body water loss. The dehydrated newborn animals would seem to reduce body water loss by decreasing the rate of glomerular filtration, but haemoconcentration and a marked increase in plasma urea are not prevented.

I wish to express my indebtedness to the Medical Research Council for a grant for the provision of technical assistance.

REFERENCES

Adolph, E. F. (1943). *Amer. J. Physiol.* **140**, 25.
Baxter, J. S. & Yoffey, J. M. (1948). *J. Anat., Lond.*, **82**, 189.
Bruner, H. D., van de Erve, J. & Carlson, A. J. (1938). *Amer. J. Physiol.* **124**, 620.
De Boer, B. (1946). *Amer. J. Physiol.* **147**, 399.
Dicker, S. E. & Heller, H. (1945). *J. Physiol.* **103**, 449.
Eichelberger, L. (1941). *J. biol. Chem.* **138**, 583.
Elkington, J. R. & Taffel, M. (1942). *J. clin. Invest.* **21**, 791.
Elkington, J. R. & Winkler, A. (1944). *J. clin. Invest.* **23**, 93.
Elkington, J. R., Winkler, A. & Danowski, T. S. (1948). *J. clin. Invest.* **27**, 74.
Gamble, J. L. (1947). *Chemical Anatomy, Physiology and Pathology of Extracellular Fluid.* Cambridge (Mass.): Harvard University Press.
Gullick, A. (1937). *Amer. J. Physiol.* **119**, 322.
Heller, H. (1941). *J. Physiol.* **99**, 3*P*.
Heller, H. (1944). *J. Physiol.* **102**, 429.
Heller, H. (1947*a*). *J. Physiol.* **106**, 28.
Heller, H. (1947*b*). *J. Physiol.* **106**, 245.
Herrington, L. P. (1940). *Amer. J. Physiol.* **119**, 322.
Lee, M. H. & Widdowson, E. M. (1937). *Biochem. J.* **31**, 2035.
McCance, R. A. & Shipp, H. L. (1931). *Biochem. J.* **25**, 449.
McCance, R. A. & Shipp, H. L. (1933). *Spec. Rep. Ser. med. Res. Coun., Lond.*, **187**, 35.
McCance, R. A. & Young, W. F. (1941). *J. Physiol.* **99**, 265.
Nadal, S. W., Pedersen, S. & Maddock, W. G. (1941). *J. clin. Invest.* **20**, 691.
Smith, H. W. (1937). *The Physiology of the kidney*, p. 65. Oxford: University Press.
Volhard, J. (1878). *Z. anal. Chem.* **17**, 482.
Whitehorn, J. C. (1921). *J. biol. Chem.* **45**, 449.
Young, W. F. & McCance, R. A. (1942). *Arch. Dis. Childh.* **17**, 65.

J. Physiol. (1949) 108, 315–339 612.816.3

EVIDENCE FOR SALTATORY CONDUCTION IN PERIPHERAL MYELINATED NERVE FIBRES

By A. F. HUXLEY and R. STÄMPFLI

From the Physiological Laboratory, University of Cambridge, and the Physiological Institute, Berne

(*Received* 12 *June* 1948)

Lillie (1925) suggested that, in myelinated nerve fibres, excitation and the processes which maintain the propagated action potential take place only at the nodes of Ranvier. On this view, the myelin is an insulator, and its function is to increase the conduction velocity by making the local circuits act at a considerable distance ahead of the active region. Much evidence in favour of this theory has accumulated since that date. Thus, many agents which cause stimulation or affect conduction have a stronger action at the nodes than in the internodal regions. This has been shown for electrical stimulation (Kubo, Ono & Toyoda, 1934; Tasaki, 1940), for blocking by electrical polarization (Erlanger & Blair, 1934; Takeuchi & Tasaki, 1942), and for blocking by various ions, ion-free solutions and narcotics (Erlanger & Blair, 1934, 1938; Tasaki, Amikura & Mizushima, 1936). Tasaki & Takeuchi (1941) obtained action currents from a short length of an isolated fibre between two narcotized regions if, and only if, the unnarcotized stretch contained a node of Ranvier. Pfaffmann (1940) obtained larger action potentials from nodes than from internodal regions.

These results all support the theory of saltatory conduction, but there are two respects in which the evidence they provide is not compelling. In the first place, they are consistent also with the hypothesis that only the axis cylinder is concerned with conduction, and that it is shielded by the myelin against external agents at all points except the nodes. In the second place, all except Pfaffmann's results refer only to the initiation or blocking of an impulse, and not directly to normal conduction. If, however, the results are taken together with the evidence that the impulse is propagated by local circuits (e.g. Hodgkin, 1937*a*, *b*, 1939; Tasaki, 1939) they provide strong evidence in favour of saltatory conduction. On the other hand, there are certain difficulties which have prevented the theory from being universally adopted. Thus, Sanders & Whitteridge (1946) conclude that conduction velocity does not depend on the

spacing of the nodes, while a simplified theory of saltatory conduction (Offner, Weinberg & Young, 1940) predicts that the velocity will increase with node spacing. Another difficulty is that, according to many authors (e.g. Maximow & Bloom, 1942; Grundfest, 1947), the myelin sheaths of fibres of the central nervous system are uninterrupted except at points where the fibres branch. If this is true, the saltatory theory cannot apply to central fibres. Bielschowsky (1928), however, insists that many authors have described interruptions in the sheaths of central fibres, and regards their existence as certain. But whichever view may be correct, this point cannot be decisive in a question which concerns peripheral nerve fibres, since it is still possible, though unlikely, that the mechanism of conduction is different in the myelinated fibres of the central and peripheral nervous systems. The other objection is likewise an indirect inference, and cannot stand against direct evidence.

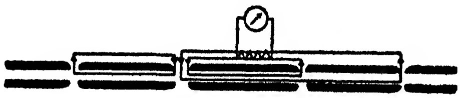


Fig. 1. Diagram illustrating principle of method.

On balance, the evidence seemed to favour the theory of saltatory conduction, but was not sufficiently direct for a certain conclusion to be reached. The object of the main experiment described in this paper (already briefly reported elsewhere, Huxley & Stämpfli, 1948) was to test the theory further by observing the distribution of current around a single fibre during the passage of an impulse. The principle of the method was suggested by Mr A. L. Hodgkin of Cambridge, who pointed out that, if current can enter or leave the axis cylinder only at the nodes of Ranvier, the current along the axis cylinder must be the same at all points in any one internode at any one moment. If a single fibre is used and the recording system passes no appreciable current, the longitudinal current outside the fibre must be equal and opposite to that in the axis cylinder. This external current can be detected by raising the external resistance over a short length of the fibre, and amplifying the potential difference which is developed across this resistance (Fig. 1). If this recording stretch can be made short compared with the length of an internode, the longitudinal current can be observed at different positions in each internode by moving the recording stretch along the fibre. Records from different positions in one internode should then be identical, while records from different internodes should be similar in form but displaced in time.

In addition, a simple experiment giving further evidence that the impulse is transmitted by local circuits is described.

METHOD

Preparation of single nerve fibres. Single myelinated fibres were isolated from the sciatic nerves of large specimens of *Rana temporaria* and *R. esculenta* by the method described by Kato (1934) and modified by one of us (Stämpfli, 1946). A few further modifications were introduced. Thus, dark-ground illumination was employed, making the fibres clearly visible whatever their direction. The oblique illumination from above that was previously used only showed up fibres that ran nearly perpendicularly to the direction of illumination. Also, the motor branch from which the fibre was to be isolated was not separated from the sensory branch with which it runs. This eliminated a difficult step in the preparation, and considerably reduced the time required.

Fibres were usually isolated for about 15 mm. After the dissection the nerve trunk was moved so that the fibre lay straight on the slide, which was placed on an ordinary microscope. The positions of the nodes of Ranvier were determined by means of the mechanical stage, and the external diameter of the fibre was measured with an eyepiece micrometer, using a 4 mm. objective and 20 × eyepiece.

The distance between adjacent nodes was fairly regular in each fibre (within ±20% except for one or two instances), but the mean distance varied from about 1·5 to 3 mm. in different fibres. It appeared not to depend on fibre diameter, which usually lay between 12 and 15 μ., while one fibre had a diameter of 18 μ.

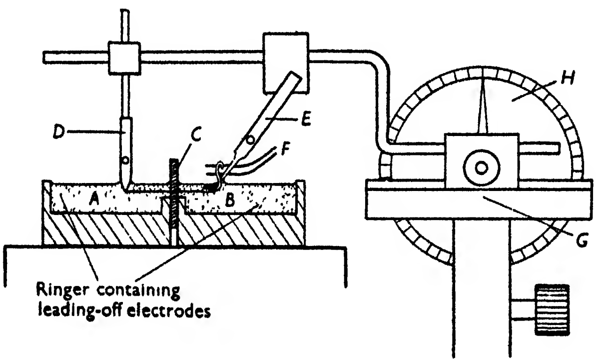


Fig. 2. General arrangement of apparatus. *A* and *B*, troughs cut in 'Perspex' blocks. *C*, partition. *D* and *E*, forceps. *F*, stimulating electrodes. *G*, micromanipulator. *H*, dial.

Apparatus. The general arrangement of the apparatus is shown in Fig. 2. The troughs *A* and *B* were cut in 'Perspex' blocks, and the channel between them was closed by a partition *C*. The fibre lay in the Ringer solution in the two troughs, passing through a hole in the partition. In order to draw the nerve fibre through the hole, a fibre of nylon or silk was pushed through, knotted round the distal end of the nerve fibre, and pulled back. The nylon or silk fibre was gripped in the forceps *D*, and the cut end of a branch of the nerve trunk was gripped in the forceps *E*. The free (central) end of the nerve trunk was lifted out of the Ringer solution and placed in contact with the stimulating electrodes *F*. These were made of silver wire, and were attached to the forceps *E*.

Both pairs of forceps were mounted on a bar carried on the horizontal movement of a micromanipulator *G*. Thus, by operating the rack and pinion, the fibre could be moved forward or back through the hole. Displacements of the fibre were measured on a dial *H* attached to the pinion shaft. The scale was divided to 0·1 mm., and intermediate values could be estimated to 0·01 mm. The forceps *E* could be moved along the bar by another rack and pinion (not shown) in order to get the fibre just stretched out. Excessive tension damaged the fibre immediately.

Trough *A* was fixed to a base-plate, while trough *B* could be moved by means of a screw. The partition was sealed in place by smearing vaseline on the opposed ends of the blocks, placing the

partition in between with its hole in line with the open ends of the troughs, and bringing the blocks together with the screw.

The partition was designed so that the resistance to current passing between the troughs outside the fibre should be fairly high (0·5–10 megohms), but that the high-resistance part of the path should extend for only 0·4–0·8 mm. along the fibre. The two ways in which this was done are shown in Fig. 3. The earlier type of partition ('oil-gap') is represented by diagram A. It consisted of two coverslips, cemented together along three of their sides with spacing pieces. A hole of diameter between 80 and 400 μ. was drilled through both, and the space between them filled with liquid paraffin. The nerve appeared to move through the holes without being damaged by touching the sides. This type of partition proved to have the following disadvantages:

(a) The resistance of the film of Ringer solution outside the fibre was greatly affected by small pieces of connective tissue, etc., adhering to the fibre.

(b) The resistance of the film was so high (of the order of 10 megohms) that stray capacities distorted the record. The distortion was prevented by inserting an external shunt, but this must have affected the distribution of current crossing the sheath in the region surrounded by oil.

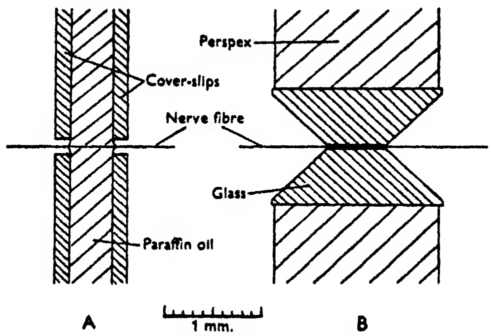


Fig. 3. Partitions. A, oil-gap. B, capillary. Approximately to scale.

(c) We tried to confirm the finding of many investigators (e.g. Kato, 1936; Erlanger & Blair, 1938) that conduction is rapidly blocked if the Ringer solution surrounding the fibre is replaced by an isotonic sugar solution, and were surprised that the fibre continued to conduct normally for about half an hour after this treatment. On the other hand, a freshly dissected fibre, which had not been in contact with paraffin oil, was blocked within 1 sec. We concluded that the oil had in some way hindered the diffusion of ions away from the film of fluid surrounding the fibre. If this was so, it was likely that the electrical properties of the fibre would also have been affected.

For these reasons the later experiments were made with partitions of the 'capillary' type shown in diagram B (Fig. 3). A piece of glass capillary tubing was drawn out so that its internal diameter was about 40 μ. Its external diameter was measured, and a hole of the same diameter was drilled through a piece of 'Perspex' sheet 1·7 mm. thick. The capillary was cemented into this hole and cut off flush with each side of the 'Perspex'. The two ends of the capillary were then opened out with a conical drill until only the central 0·5 mm. or so had the original diameter. This type of partition had a resistance of about 0·5 megohm, giving a rather low signal/noise ratio. Capillaries of smaller diameter were tried, but the fibres appeared to be damaged in passing through them.

Electrical recording system. We used the amplifier and cathode-ray oscillograph described by Hodgkin & Huxley (1945). This was built as a direct-coupled balanced amplifier. Since we were concerned only with rapid changes of potential, one of the stages was coupled by resistance and capacity with a time constant of about 0·5 sec. It was also found unnecessary to use it as a balanced instrument, and one of the inputs was connected to earth throughout these experiments.

The input stage was a cathode follower, placed with the grid cap of the valve within 5 cm. of the preparation. Fig. 4 A shows the circuit finally employed when the oil-gap partition was in use.

The effects of stray capacities were reduced by the following means. The capacity of the control grid to earth through the screen grid and anode was reduced by connecting the screen grid to the cathode through an h.t. battery of the appropriate voltage. The potential of the screen grid was thus made to follow the changes in potential of the control grid, so that practically no current passed through the capacity between them. The effect of the capacity of the live electrode to the stand and other earthed objects was similarly reduced by connecting the stand, micromanipulator, etc., to the cathode instead of to earth. Finally, the preparation was shunted by a resistance of 2·7 megohms, which brought the time constant down to about 20 μsec. Further reduction of the shunt resistance did not appear to affect the shape of the action-current record.

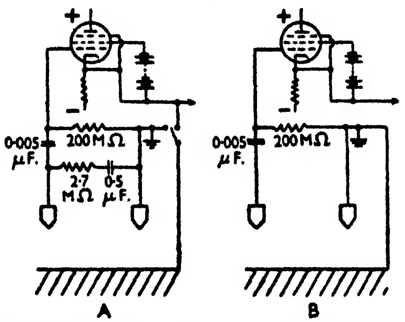


Fig. 4. Input circuits used (A) with oil-gap and (B) with capillary type of partition.

Condensers were inserted in the positions shown to prevent steady currents from passing through the preparation as a result of either the grid current of the valve or the residual potential difference between the electrodes. The grid leak was 200 megohms.

Fig. 4 B shows the input circuit used with the capillary partition. The resistance of the preparation was only about 0·5 megohm in this case, so that the stray capacities did not produce serious effects.

The electrodes were pieces of silver sheet, coated electrolytically with silver chloride. The earth electrode was fixed in trough *B*, while the leading-off electrode was fixed in trough *A* (Fig. 2).

The nerve trunk was stimulated by short thyratron discharges at a frequency of about 40 shocks/min. throughout the experiment. The strength of the shocks was adjusted to about twice the threshold for the isolated fibre.

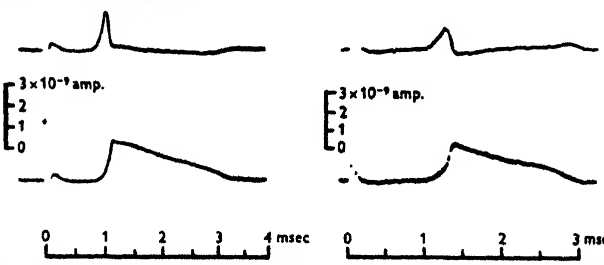


Fig. 5. Records of longitudinal current taken (left) with oil-gap and (right) with capillary type of partition. In each case, upper record taken just proximal to, and lower record just distal to, last working node.

RESULTS

Shape of action-current records. Typical records of the action current at about the middle of an internode are shown in Fig. 5. The left-hand pair of records was obtained with the 'oil-gap', the right-hand with the 'capillary' partition.

The upper record in each case is taken from a point on the fibre which is far enough from the damaged distal end to give a normal action current. The lower records are from points beyond which there is no activity, and the impulse is conducted decrementally for a few millimetres by electrotonic spread. On the theory of saltatory conduction, it would be said that, in the upper records, the node at each side of the recording stretch becomes active, while in the lower records the node of the proximal side of the recording stretch becomes active while that of the distal side does not.

It will be seen that these records are very similar in shape, amplitude and duration to those published by Tasaki & Takeuchi (1941). The upper records correspond to their 'binodal action current' and the lower records to their 'mononodal action current'.

Action currents at various positions in an internode. Fig. 6 shows a series of action-current records taken at different positions along a fibre with the capillary partition. The positions are chosen so that there are three records from each internode, one as near as possible to its proximal end, one near the middle, and one as near as possible to its distal end. There is never a node of Ranvier within the recording stretch.

It will be seen that the three records from any one internode are practically synchronous, while records from different internodes are displaced in time. This is also shown in Fig. 7, where the times of certain features of the first phase of the record, measured from the shock artefact, are plotted against distance. These conduction times increase discontinuously at certain definite positions on the fibre. This was an invariable finding, and the spacing between the discontinuities always agreed with the measured spacing between the nodes. In a number of cases the nodes were located with a microscope while the fibre was in the apparatus and records were being taken. It was then found that the discontinuities occurred when a node was in the recording stretch. We shall assume that this was also the case in the experiments where the nodes were not located visually after the fibre had been mounted in the apparatus.

The velocity of conduction in the isolated part of the fibre is the reciprocal of the mean slope of either of the two lowest graphs in Fig. 7. It is found to be 23·2 m./sec. The detailed analysis described later was carried out on records obtained from this fibre and from three others which gave velocities of 22·2, 24·3 and 23·1 m./sec. These values are normal for frog fibres of 12–15 μ. diameter, at temperatures of 18–20° C. (Erlanger & Gasser, 1937), indicating that the fibres cannot have been seriously damaged by the dissection and other experimental procedures. Most other fibres gave somewhat lower velocities (not less than 12 m./sec.). It is possible that these had been damaged, but in all qualitative respects the results they gave were similar to those described here.

So far, this is what would be expected if current entered or left the axis cylinder only at the nodes. But if that were actually the case, the records would be identical in shape and amplitude, as well as in time, at different positions in one internode. The records in Fig. 6 show that this is not the case. The amplitude

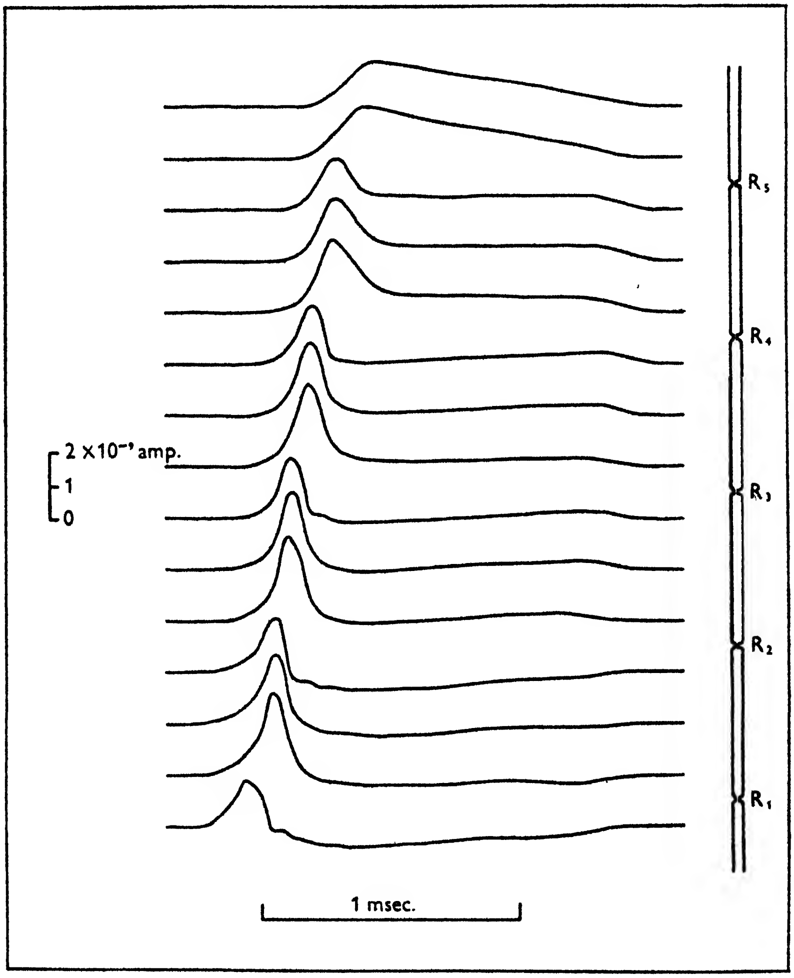


Fig. 6. Tracings of records obtained at a series of positions along one fibre, with capillary partition. Stimulus artefact has been subtracted. Diagram of fibre on right-hand side shows position where each record was taken.

of the first phase decreases from the proximal towards the distal end of each internode. This is better seen in the upper graph of Fig. 7. Also, the shape of the record is different at the different positions. The peak of the first phase is sharpest at the proximal end of each internode, while the angle where the record becomes flat at the end of the first phase is sharpest at the distal end of

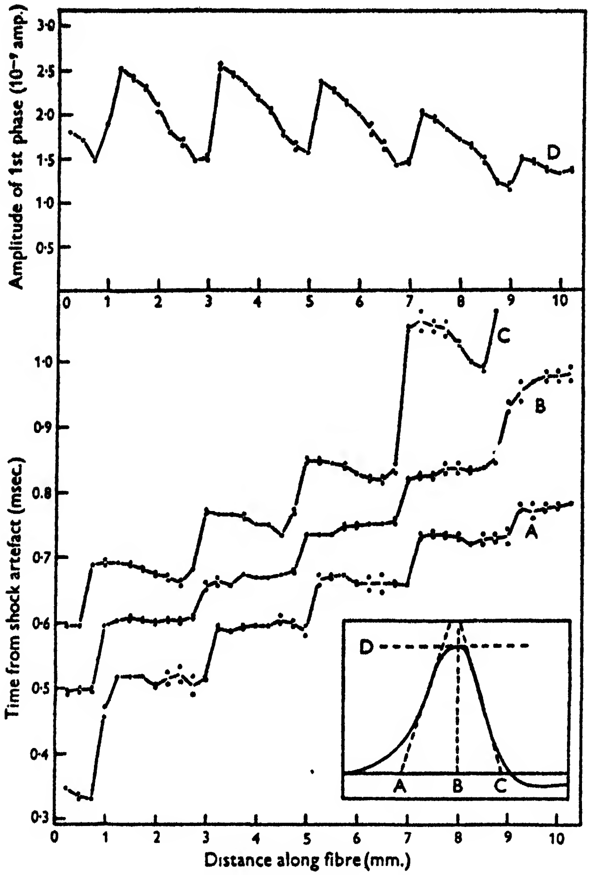


Fig. 7. Lower section: conduction times of (A) upstroke, (B) peak, and (C) downstroke of first phase of record, plotted against distance along fibre. Upper section: amplitude of first phase, plotted against distance along fibre. Inset: diagram of first phase showing how each quantity was measured. From same records as Fig. 6.

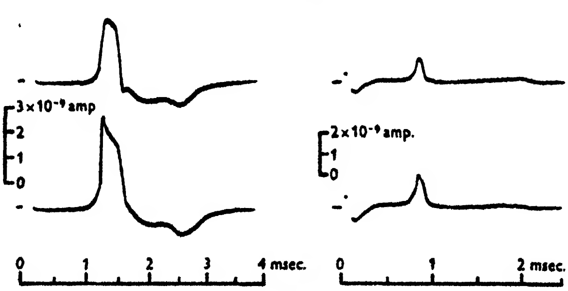


Fig. 8. Longitudinal current at proximal end (lower record) and distal end (upper record) of one internode. Left-hand pair of records obtained with oil-gap, right-hand with capillary type of partition.

the internode. Two pairs of records showing this difference clearly are shown in Fig. 8. These differences between the records obtained at different positions within one internode mean that some current does pass through the myelin sheath. Without further analysis these results are therefore not unequivocal evidence in favour of saltatory conduction. It might be, for instance, that conduction is extremely rapid in each internode, and that a delay occurs at each node because the membrane capacity and conductance are higher there than in the internodes.

The analysis required to clear up this point is carried out in the section entitled 'Determination of membrane current', and shows that the current through the myelin can be explained as a passive current driven through a resistance and a capacity in parallel by the change in potential in the axis cylinder. On this basis the fact that the graphs in Fig. 7 are not horizontal straight lines in each internode can be interpreted as follows. As regards the graph of amplitudes the potential in the axis cylinder is rising and causing current to flow outwards through the electrostatic capacity of the myelin during the first phase of the current record. The logitudinal current is directed forwards in the axis cylinder, so that outward current through the myelin makes the amplitude of the longitudinal current decrease from the proximal to the distal end of each internode.

The graphs of times, with the surprising feature that the descending phase occurs earlier at the distal than at the proximal end of the internode, are best understood by considering the spread of longitudinal current due to the potential change at one node. The rapid rise of potential in the axis cylinder at the node causes, in the axis cylinder of the internode on the distal side, an increase in forward current whose peak is roughly indicated by the peak of the first phase of the record. In the more proximal internode, however, it causes a decrease in the forward current whose peak is given approximately by the end of the first phase, or by the time which is plotted as graph C. Thus, graph C in one internode and graph B in the next more distal internode represent different aspects of the same disturbance spreading symmetrically from the node separating them. This spread takes place with a finite velocity (not necessarily constant), so that graph B becomes later, and graph C earlier, towards the distal end of each internode.

Results when recording stretch contains a node. Consider first the results that would be expected when the recording stretch contains a node, on the hypothesis that current enters and leaves the axis cylinder principally at the nodes. The potential difference across the recording stretch is built up partly by the current in the more proximal of the two internodes separated by this node, and partly by that in the more distal one. As a simplified case we shall first assume that the longitudinal current is the same at all points in one internode at any one moment. The situation is illustrated by Fig. 9.

Let s = length of recording stretch,
y = distance of node from proximal end of recording stretch,
i_a = longitudinal current in axis cylinder of more proximal internode,
i_b = longitudinal current in axis cylinder of more distal internode,
r_1 = resistance per unit length of fluid surrounding fibre,
v = potential on distal side − potential on proximal side.

Then v = potential drop between C and B + potential drop between B and A

$$= r_1 (s-y)\, i_b + r_1 y i_a$$
$$= r_1 s\,(i_b + (i_a - i_b)\, y/s).$$

v is therefore a linear function of y, and is equal to $r_1 s i_b$ when $y=0$, and to $r_1 s i_a$ when $y=s$. If the recording stretch is shunted by a resistance, it is easy to show that the same result holds, except that the coefficient $r_1 s$ is replaced by the parallel resistance of $r_1 s$ and the shunt.

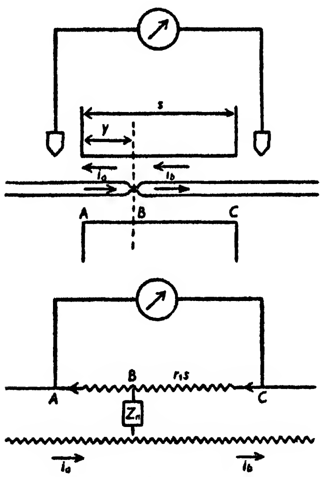


Fig. 9.

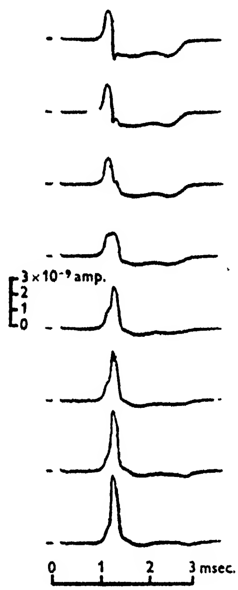


Fig. 10.

Fig. 9. Diagram of situation when recording stretch contains a node. Z_n = impedance of membrane at node. For meaning of other symbols, see text. Proximal end of fibre is to the left.

Fig. 10. Records taken as a node passed through the recording stretch. Fibre moved 0·1 mm. between successive records. Oil-gap type of partition.

This relation cannot be expected to hold exactly in a real case for three reasons. The first is that some current does cross the myelin, so that i_a and i_b are not constants, but depend on distance from the node. The second is that unless r_1 is small compared with the resistance per unit length of the axis cylinder, the values of i_a and i_b at given positions in the fibre will change as the fibre is drawn through the recording stretch. The third is that r_1 may not be the same at all points on the fibre.

The first of these factors is probably unimportant. If i_a and i_b can be sufficiently represented as linear functions of the distance x along the fibre, then the expression for v contains a term in $y(s-y)$ proportional to $(di_a/dx - di_b/dx)$. The values of these differential coefficients can be obtained from records taken in the internodes, and calculation shows that the resulting deviation from linearity of the relation between v and y is negligible.

The second factor probably has an appreciable effect on records taken with the oil-gap, but not on those taken with the capillary, in which the value of r_1 is of the order of $\frac{1}{30}$ of the resistance per unit length of the axis cylinder.

The third factor probably also caused appreciable errors with the oil-gap but not with the capillary. With the oil-gap, the resistance of the external fluid film was affected by local variation in fibre diameter, and, probably more important, by connective tissue fibrils, etc., adhering to the fibre. This factor would be expected to cause irregularities in the relation between observed potential and distance also in the internodes; this is sometimes detectable in records taken with the oil-gap, but not with the capillary.

These sources of error are therefore probably not serious, but may cause deviations from the quantitative predictions of the simple theory when the oil-gap partition is used. We should thus expect that, as the node goes from one side of the recording stretch to the other, the measured potential will change steadily from its value in the proximal to that in the distal internode, but that the change may not be exactly linear, especially in records taken with the oil-gap partition.

Fig. 10 shows a series of records taken at various positions as a node of Ranvier passed through the recording stretch. In order to see whether the transition between the two forms of action potential takes place as predicted,

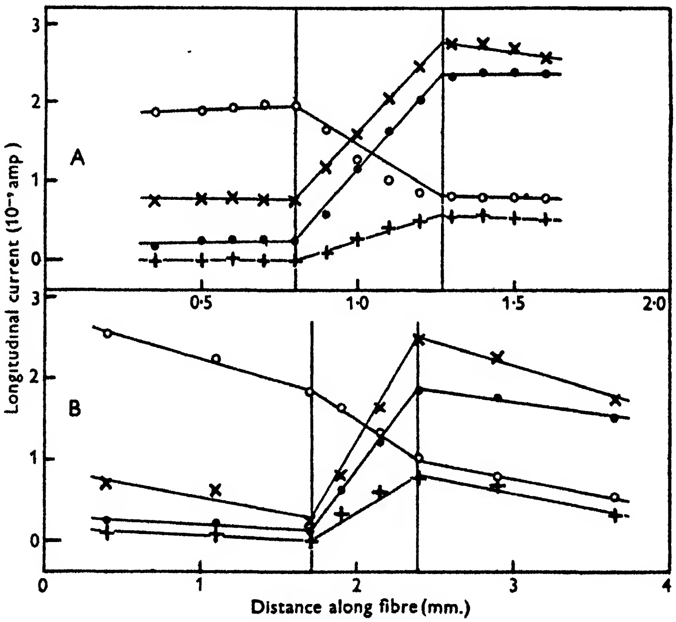


Fig. 11. Longitudinal current plotted against distance along fibre, as a node passes through recording stretch. Each graph corresponds to a constant time after the stimulus. A, oil-gap; B, capillary type of partition. ○—○, near peak of first phase in proximal internode; ×—×, near peak of first phase in distal internode; ●—●, during downstroke of first phase in distal internode; +—+, near end of first phase in distal internode. In A, scale of ordinates is only approximate.

the observed potential is plotted in Fig. 11 against distance along the fibre. Each graph in Fig. 11 corresponds to a particular time after the stimulus. The ordinates are the deflexions at that time in the records taken at varying

positions as the fibre was drawn through the recording stretch. The records from which Fig. 11 A was made were taken with the oil-gap, at intervals of 0·1 mm. The exact length of the recording stretch could not be determined, as the menisci of the oil could not be seen. The positions at which the node enters and leaves the recording stretch are, however, clearly seen on the graphs, and on the simple theory the points in between these should lie on the straight lines joining the ordinates at those positions. Fig. 11 B was constructed from records taken with the capillary partition at intervals of 0·25 mm. The capillary was 0·60 mm. long, but end-effects would be expected to increase its apparent length to about 0·68 mm. The vertical lines in the figure are drawn at this distance apart, and on the simple theory, the points between them should lie on the straight lines which have been drawn, joining the ordinates on the vertical lines. By good fortune, records were obtained when the node was only just outside each end of the recording stretch. It is evident fron the graphs that the potentials at these positions are unaffected by the proximity of the node to the recording stretch.

In both cases the prediction is fulfilled as closely as could be expected from the approximations in the theory and the errors of measurement. This is evidence that the large currents which are shown to enter and leave the axis cylinder in the neighbourhood of the nodes do so within a distance which is short compared with the recording stretch.

Determination of membrane current. The potential recorded by the method described in this paper is proportional to the average, taken over the recording stretch, of the longitudinal current in the axis cylinder. This average is equal to the value of the current at the middle of the stretch if the longitudinal current can be adequately represented, over the stretch, as a linear function of distance along the fibre. This condition is certainly fulfilled so long as the recording stretch does not contain a node of Ranvier. With this proviso, we can therefore say that the observed potential is proportional to the longitudinal current in the axis cylinder at the middle of the recording stretch. If this current is found to be different at two positions on the fibre, at the same moment, then the difference between these currents must have entered the axis cylinder (or left it, as the case may be) in between these positions. We shall refer to this difference as the 'membrane current'.

Thus we can find the current entering or leaving the axis cylinder by taking the difference between the potentials recorded at two nearby positions on the fibre. With our apparatus it was not possible to lead off potentials simultaneously from two stretches of the nerve fibre. We therefore took a record at one position, moved the fibre, and took another record. We then took the difference between the potentials in these two records at the same time after the stimulus. This procedure may have introduced some errors, since the action currents at any one position on the fibre may not have been identical when the two records were taken. In particular, with the oil-gap method, the position of the recording stretch on the fibre probably affected the current distribution. This objection probably does not arise with the capillary method, since the resistance of the fluid

outside the fibre in the recording stretch was small compared with that of the axis cylinder. The results obtained with the two types of partition are very similar, but we shall rely chiefly on the capillary method both because of this objection to the oil-gap method, and because of the other objections mentioned under the heading 'Apparatus'.

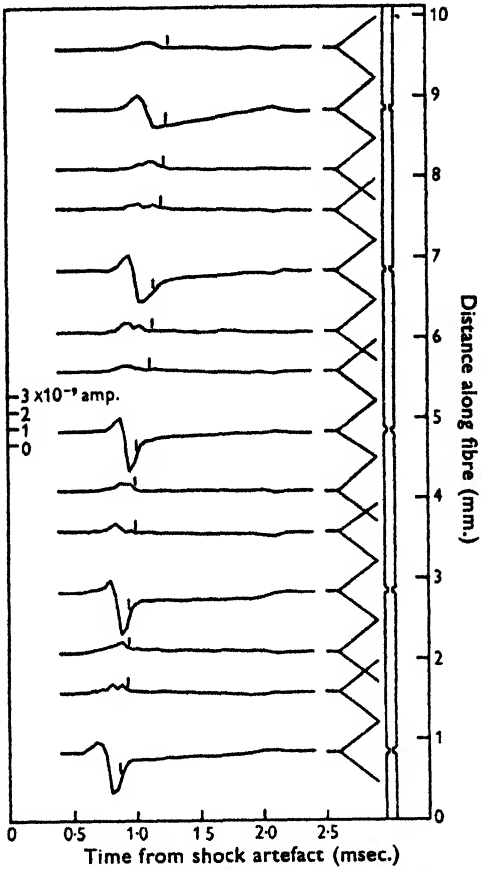


Fig. 12. Membrane currents. Each curve shows the difference between the longitudinal currents at two points 0·75 mm. apart on the fibre. The positions of those two points relative to the nodes is indicated on the diagrammatic fibre on the right. The vertical mark above each graph shows the time when the change in membrane potential reached its peak at that position on the fibre. Outward current is plotted upwards.

Fig. 12 shows a series of results obtained with the capillary partition. It is at once obvious that each graph which refers to a stretch of the fibre containing a node of Ranvier has a large inward component, while the other graphs do not. These curves correspond to the records obtained with tripolar recording by Tasaki & Takeuchi (1941 for nodal, 1942 for internodal stretch), as regards both the principle of the method and the form of the results. With the apparatus used by those authors, however, it was not possible to obtain series of records from different points on the same fibre. Also their recording stretches were

longer than ours, so that a record containing the membrane current at a node contained also the current through the myelin sheath of a greater length of the fibre. This makes the first, outward, current pulse larger, and the second, inward, current pulse smaller and of shorter duration, in Tasaki & Takeuchi's records than in ours. In our records, the nodal stretch includes a length of fibre equal to that from which each internodal record was obtained. The current through the myelin sheath of this stretch must therefore be nearly equal to the mean of the internodal currents observed on either side of the nodal stretch. The current entering or leaving the axis cylinder at the node itself could be obtained by subtracting this mean current from the total current observed in the nodal stretch. This procedure would evidently decrease further the size of the first, outward, pulse of current, and increase the size of the second, inward, pulse, without appreciably altering the times at which they occur.

In order to interpret these curves it is necessary to know approximately the time course of the potential difference between axis cylinder and external fluid at each position where the membrane current is measured.

We did not obtain records of this potential directly, but its form can be obtained by integrating with respect to distance the recorded action current at a constant time after the stimulus. The fluid outside the fibre between the recording stretch and the distal end was practically equipotential, so that

$$\partial V_m/\partial x = r_2 i,$$

where V_m = potential difference across myelin sheath = potential in surrounding fluid − potential in axis cylinder, V_0 = resting value of V_m, x = distance along the fibre, r_2 = resistance per unit length of axis cylinder, and i = observed current = deviation of longitudinal current in axis cylinder from its resting value.

Hence
$$V_m - V_0 = r_2 \int i\, dx.$$

The integration should be taken from a point where no action potential is detectable to the position where the result is required.

This integration was carried out numerically on several sets of records. The conclusions were similar in all cases, but the best results were obtained with the records from which Figs. 6 and 12 are constructed. In this case there was some decremental spread of action current beyond the position where the most distal record was taken, and an allowance for this had to be estimated and added to the result of integration. The main conclusion which is drawn, however, is quite independent of these estimates.

The time at which the potential change across the membrane reached its maximum was read from the curve obtained for each position, and is marked on the corresponding membrane current curve in Fig. 12.

Each of the curves in Fig. 12 taken from a length of the fibre which does not include a node is closely similar to the current which would flow if the calculated membrane potential change were applied to a resistance and capacity in parallel. In the rising phase, the currents through both elements are outward, and add to give the definite outward pulse which is seen in the records. During

the falling phase, however, the current through the capacity is inward, while that through the resistance is still outward. The net current in this phase is therefore small and may be either inward or outward, according to the rate at which the potential falls and the size of the product of the values of the resistance and capacity.

All these 'internodal' records can be explained in this way. In each record from a length of fibre which contains a node, however, there is a large inward pulse of current which begins and indeed reaches its maximum while the internal potential is still rising. This cannot be explained as a passive current being driven through any ordinary circuit element by the change in membrane potential, and must be regarded as current produced 'actively' by the nerve.

Determination of resistance and capacity of myelin. The time course both of the current through the myelin sheath and of the change in the potential difference across it were determined by the methods described in the previous section. The results suggested that the myelin behaves like a membrane with finite conductance and capacitance in parallel. If it is assumed that this interpretation is correct, and, further, that the conductance and capacitance are linear and constant at each point on a fibre, it should be possible to calculate the values of these circuit elements at each position where the current and potential difference curves have been found. This was done at a series of points on each of four fibres. The method used was as follows:

Let i_m = current entering axis cylinder per unit area of myelin,
G_m = conductance of myelin per unit area,
C_m = capacitance of myelin per unit area.

V_m, V_0, and r_2 have the same significance as in the preceding section.

Then
$$i_m = G_m (V_m - V_0) + C_m \frac{\partial V_m}{\partial t}.$$

Integrating this with respect to time,

$$\int_{t_1}^{t_2} i_m \, dt = G_m \int_{t_1}^{t_2} (V_m - V_0)\, dt + C_m \left[(V_m)_{t_2} - (V_m)_{t_1} \right].$$

To find the conductance, the integration was taken from the beginning of the action potential ('foot') to the time when the potential difference had returned to its initial value ('end'). Then

$$(V_m)_{t_1} = (V_m)_{t_2} \quad \text{and} \quad G_m = \int_{\text{foot}}^{\text{end}} i_m \, dt \Big/ \int_{\text{foot}}^{\text{end}} (V_m - V_0)\, dt.$$

To find the capacitance, the integration was taken from the beginning of the action potential to the time when the potential change was a maximum ('peak'). Then

$$(V_m)_{t_2} - (V_m)_{t_1} = \text{height of action potential},$$

and
$$C_m = \frac{\int_{\text{foot}}^{\text{peak}} i_m \, dt - G_m \int_{\text{foot}}^{\text{peak}} (V_m - V_0)\, dt}{\text{height of action potential}}.$$

The value used for G_m in this equation was that determined at the same position on the fibre.

Membrane currents were obtained by differencing the observed longitudinal current, as described in the previous section. Each value was divided by the area of the myelin from which the current was obtained in order to give i_m, the membrane current per unit area.

The values of $(V_m - V_0)$ and of the height of the action potential were determined by the integration method described in the previous section. The results of this method are proportional to the value taken for r_2, the longitudinal resistance per unit length of the axis cylinder. This was not measured, and the value used was calculated from the dimensions of the axis cylinder, using the specific resistance of Ringer solution. The values of the potential change thus obtained are too small by the factor α by which the specific resistance of the axoplasm exceeds that of Ringer solution. The values of conductance and capacitance must therefore be divided by this unknown factor α. The specific resistance of Ringer solution was taken as 94 ohm-cm. at 18° C., 92 ohm-cm at 19° C. and 90 ohm-cm. at 20° C. These values were based on data kindly provided by Dr B. Katz, of University College, London.

The results of this analysis are given in Table 1, together with other particulars of the data on which it was carried out.

TABLE 1. Results of complete analysis of membrane currents of four fibres. α = (specific resistance of axoplasm)/(specific resistance of Ringer solution, taken as 90 ohm-cm. at 20° C.)

Date of experiment ...	5. xii. 47	17. xii. 47	4. i. 48	7. i. 48
Type of partition used ...	Oil-gap	Oil-gap	Capillary	Capillary
Species of frog ...	*Esculenta*	*Temporaria*	*Esculenta*	*Temporaria*
External diameter of fibre (μ.)	14·5	15·0	14·5	12·0
Thickness of myelin assumed (μ.)	2·0	2·0	2·0	1·5
Temperature (° C.)	18	19	20	20
Conduction velocity (m./sec.)	22·2	24·3	23·1	23·2
Highest action potential (mV.)	106α	93α	79α	109α
Mean capacity of myelin sheath (μF./cm.^{2})	0·0023/α	0·0030/α	0·0035/α	0·0035/α
Dielectric constant of myelin	5·1/α	6·8/α	7·9/α	6·0/α
Resistance of myelin sheath (megohms-cm.^{2}) (reciprocal of mean conductance)	0·082α	0·109α	0·167α	0·158α
Specific resistance of myelin (ohm-cm. × 10^8)	4·2α	5·5α	8·4α	10·5α
No. of positions on fibre where resistance and capacity were measured	5	8	5	6
Standard error of mean capacity (% of mean)	34	10	9	3
Standard error of mean conductance (% of mean)	18	12	12	30

Experiment to test local circuit theory. Two microscope slides were supported end to end on insulating blocks screwed to an earthed base-plate. A single-fibre preparation was chosen in which stimulation of the nerve trunk, proximal to the isolated stretch, caused a visible contraction in the muscle, which was left attached to the nerve. The preparation was laid in a pool of Ringer solution on the slides, so that the muscle lay on one slide and the nerve trunk on the other, with the isolated fibre crossing the junction. The slides were drawn apart, making a gap of 1–2 mm. which was bridged by the isolated fibre. Care was taken that the part of the fibre in the air-gap should not contain a node of Ranvier. The pools of Ringer solution on the two slides could also be connected by laying a thread, moistened with Ringer solution, across the gap. The nerve trunk was stimulated by means of galvanic forceps. It was found that the muscle twitched when the nerve was stimulated if, but only if, the thread connecting the fluids on the two sides of the gap was in place.

The only effect of putting the thread in place was to make an electrical

connexion between the two pools of Ringer solution. Conduction was thus impossible if the longitudinal resistance outside the fibre was above a certain value. This demonstrates that the transmission of the nervous impulse depends on currents flowing outside the myelin sheath, the circuit being presumably completed by the axis cylinder. In non-medullated nerve fibres, whose properties are the same at all points along their length, a change in external resistance affects only the velocity of conduction, and transmission should still be possible however high the external resistance. In a discontinuous system this is not the case. The explanation of this difference of behaviour is as follows.

In a continuous system, the velocity is proportional to $1/(r_1+r_2)^{\frac{1}{2}}$, where r_1 = resistance per unit length of external fluid, and r_2 = resistance per unit length of the axis cylinder. This relation has been derived for certain particular sets of assumptions by Rushton (1937) and by Offner *et al.* (1940), but can also be shown to be a direct consequence of uniform propagation by local circuits, as mentioned by Hodgkin (1947). The time course of the potential change is unaltered, so that its length scale is also proportional to $1/(r_1+r_2)^{\frac{1}{2}}$. The potential gradient is therefore increased in proportion to $(r_1+r_2)^{\frac{1}{2}}$, and the longitudinal current, which is equal to (potential gradient)/(r_1+r_2), is reduced in proportion to $1/(r_1+r_2)^{\frac{1}{2}}$. The length of nerve in front of the active region which has to be depolarized in order to excite it is, however, also reduced in the same ratio, so that the current is sufficient. But in a discontinuous system the membrane (at a node) which has to be depolarized has a fixed capacity and conductance. Its distance from the active node is also fixed, so that the longitudinal current is proportional to $1/(r_1+r_2)$. If r_1 is increased sufficiently, this current will therefore become insufficient to excite.

The experimental result that an increase of external resistance can cause a block might therefore be taken as evidence not only that the impulse is transmitted by local circuits, but also that the system is discontinuous. This point will not be pressed, however, for two reasons. In the first place, it is possible that the part of the fibre which was in air might have been damaged, so that the fibre was made discontinuous by the experimental conditions. In the second place, although a continuous system could in principle conduct in a region where the external resistance was indefinitely high but uniform, it might block at a point where the external resistance drops suddenly from a high to a low value.

DISCUSSION

Evidence for saltatory conduction. The results described in this paper include three more or less independent pieces of evidence in favour of the view that 'activity' takes place only at the nodes of Ranvier.

(*a*) The amplitude and time of occurrence of the main action current wave change discontinuously at each node of Ranvier.

(*b*) The membrane current through the myelin can be explained as a passive current through a resistance and capacity in parallel, but the membrane current at a node of Ranvier has a component which cannot be so explained.

(*c*) Conduction can be blocked by raising the external resistance.

It was pointed out in the 'Results' section that alternative explanations could be produced for observations (*a*) and (*c*). We do not believe that these alternative explanations are correct, but the fact that they exist reduces the value of these pieces of evidence. We shall therefore rely chiefly on the analysis of the distribution of membrane current during the passage of an impulse.

In the region covered by the nerve impulse, the interior of a nerve fibre is more positively charged relative to the exterior than when it is in its normal state. The sheath of the fibre has a finite conductance, so that this charge tends to leak away. For non-decremental conduction, this leakage must be replaced, and the process by which this takes place is called 'activity'. It occurs predominantly in the front of the region carrying the charge, so that the impulse moves forward. If this inward transfer of positive charge could be detected as such, it would be the most direct criterion of activity. But we can only measure the total current entering or leaving the axis cylinder (membrane current), and this contains the currents through the conductance and capacitance of the sheath as well as the current due to 'activity'. If, therefore, we are to locate the regions in a nerve fibre where 'activity' takes place by observing the membrane current, we must first deduce relations in terms of membrane current which are characteristic of 'activity'.

We could evidently say that a region of a nerve fibre does not take part in 'activity' if all the currents in it during an impulse can be explained by the observed potential change acting on known passive conductances and capacities. The resistance and capacity of the myelin sheath have not yet been measured, so that the most that could be said now is that there is no evidence of activity if the currents during an impulse can be explained by the observed potential change acting on plausible values of resistance (not necessarily linear or constant) and capacity. By 'plausible' is meant, for instance, that the capacity should not be so low as to imply an improbable value for the dielectric constant of the myelin.

It would, however, be unsatisfactory to locate 'activity' solely by the failure to satisfy this condition. We shall therefore try to find a characteristic way in which the membrane current of an 'active' region differs from the current to be expected in the absence of activity.

Consider the membrane current to be expected during non-decremental conduction of an impulse in a core conductor whose sheath has resistance and capacity. These passive elements are assumed to be in parallel with each other and with the mechanism which produces the 'active' current; this schematic circuit is not fundamentally different from that which represents 'activity' as a change in the e.m.f. in series with the resistance, but is somewhat more general.

Let x = distance along fibre,

r_1 = longitudinal resistance per unit length of fluid around fibre,

r_2 = longitudinal resistance per unit length of axis cylinder,

i_1 = longitudinal current outside fibre, positive in direction of increasing x,

i_2 = longitudinal current in axis cylinder, positive in direction of increasing x,

V_1 = potential in fluid surrounding fibre,
V_2 = potential in axis cylinder,
G_m = conductance of sheath per unit length,
C_m = capacitance of sheath per unit length,
i_m = current entering axis cylinder per unit length,
V_m = potential difference across sheath $= V_1 - V_2$,
V_0 = resting value of V_m,
u = velocity of conduction, assumed positive so that impulse travels in direction of increasing x.

We shall take first the case of a fibre whose properties are uniform along its length. We assume that during the action potential, $(V_m - V_0)$ is negative and has only one minimum. Then

$$r_1 i_m = -r_1 \partial i_1/\partial x = \partial^2 V_1/\partial x^2 \quad \text{and} \quad r_2 i_m = r_2 \partial i_2/\partial x = -\partial^2 V_2/\partial x^2,$$

so that
$$\partial^2 V_m/\partial x^2 = \partial^2 V_1/\partial x^2 - \partial^2 V_2/\partial x^2 = (r_1 + r_2)\, i_m.$$

During steady conduction, $V_m = f(t - x/u)$, so that

$$\partial^2 V_m/\partial x^2 = 1/u^2 . \partial^2 V_m/\partial t^2 \quad \text{and} \quad i_m = 1/(r_1 + r_2)\, u^2 . \partial^2 V_m/\partial t^2.$$

The membrane current is therefore outward when the graph of V_m against t (the action potential plotted with its peak downwards) has a downward curvature, and inward when it has an upward curvature. The curvature begins downward, but must become upward before the peak of the potential change in order for $\partial V_m/\partial t$ to be zero at the peak. Hence, there must be inward membrane current before the peak of the potential change. During the whole of this phase the currents through the membrane conductance and capacitance are both outward, since both $(V_m - V_0)$ and $\partial V_m/\partial t$ are negative. Hence the inward membrane current must be produced by some 'active' process. This active process must, of course, produce a greater inward current than this, since it is only the excess over the currents through the conductance and capacitance which can be observed as 'membrane current'.

Now consider the more difficult case of a nerve fibre in which activity takes place only at certain small areas of the sheath, which we shall refer to as 'nodes'. We shall assume as before that $(V_m - V_0)$ is negative during the impulse and has only one minimum, and that the time course of the potential change is the same at each position where activity occurs, though occurring later the greater the value of x. The distribution of potential along the fibre will then be roughly as shown in Fig. 13. In each internodal stretch,

$$\partial^2 V_m/\partial x^2 = (r_1 + r_2)\, i_m = (r_1 + r_2)\,\{G_m(V_m - V_0) + C_m \partial V_m/\partial t\},$$

which must be negative throughout the rising phase, so that the curvature of the graph is downward between each two successive nodes.

When the value of V_m at a particular node is at its minimum, and for a finite time before and after, the point on Fig. 13 representing it must lie below the straight line joining the points representing the values of V_m at the neighbouring nodes. Hence $\partial V_m/\partial x$ must be algebraically greater just beyond the node than just before it, and the same is true of i_2, since $i_2 = 1/(r_1+r_2).\partial V_m/\partial x$. The membrane current at the node itself must therefore be inward, by Kirchhoff's first law. Hence, at the points where activity occurs, the membrane current becomes inward before the peak of the potential change at that point, while at all other points this is not the case.

This argument depends on the assumption that the amount of activity is the same at all points where activity occurs. But if, at points between the nodes, a slight degree of activity existed which was insufficient to make the membrane current inward before the peak of the potential change, then the argument and its conclusion would be unchanged. We can therefore conclude that points

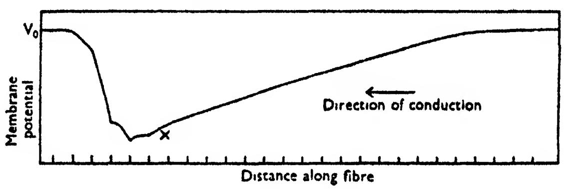


Fig. 13. Diagram showing form of distribution of membrane potential along a nerve fibre with nodes. Positions of nodes marked on horizontal axis. The part of the curve to the left of the point X was obtained by the integration method from actual records of longitudinal current.

must exist where the membrane current becomes inward before the peak of the potential change, and that these are the points where at least the main part of the activity is located. It was shown in the 'Results' section that the membrane current at each node of Ranvier behaved exactly in this way, while that through the myelin sheath did not. We take this as proof that the main part of the 'activity' in these nerve fibres occurs at the nodes of Ranvier.

The question remains whether or not a slight amount of activity occurs in the internodal regions. The 'active current', if any, would be sufficient only to reduce the losses, and not to contribute directly to the current which passes forward in the axis cylinder to depolarize the sheath ahead of the active region. The only criterion we can apply in this case is whether or not the membrane current through the myelin can be explained without postulating activity. As stated in the 'Results' section, the membrane current agrees qualitatively with the current which would flow if the membrane potential change were applied to a resistance and capacity in parallel. The agreement is in fact closer than was indicated at that point. The two peaks frequently seen in the membrane current curves during the rising phase of the potential change correspond to two detectable maxima in the rate of rise of potential, as determined by our

integration method. This agrees with the finding of Tasaki & Takeuchi (1942) that each of these peaks is due to activity at the node at one end of the internode under observation, and not in the internode itself. Also, the small pulse of inward current at the very end of the impulse corresponds to a rapid fall in the calculated potential curves.

Inward current due to activity would make the conductance of the myelin appear lower than its true value. If, as would be expected, the activity occurred chiefly in the rising phase of the potential change, it would also reduce the apparent value of the capacity. It would therefore be evidence of 'activity' in the myelin if the apparent capacity or conductance of the myelin were improbably low for a passive membrane of its thickness. Values of these quantities deduced from our records, and given in Table 1, are uncertain in that they include an unknown factor α, the ratio of the specific resistance of the axoplasm to that of the surrounding medium. The value of this ratio has been determined for axons of certain invertebrates; it is about 1·4 in the giant axon of *Loligo* (Cole & Hodgkin, 1939), 3·0 in *Homarus* axons (Hodgkin & Rushton, 1946) and 4·5 in *Carcinus* axons (Hodgkin, 1947). A value between 1·4 and 1·8 seems likely for the fibres used in our experiments; this would imply that the action potential is about 130–170 mV. in amplitude (Table 1). But even if α is as high as 2·2, and the action potential is about 210 mV. the specific resistance of the myelin is only about 2×10^9 ohm-cm., which does not seem improbably high, and the apparent dielectric constant is about 3. The value found by our method for the capacity, and hence that for the dielectric constant, would be considerably affected if the resistance of the myelin sheath changed during the impulse or were non-linear. If either of these effects existed and were in the same direction as was observed in *Loligo* by Cole & Curtis (1939) and Cole & Baker (1941), it would make the capacity appear smaller than its true value. Thus, our results make it unlikely that the dielectric constant of the myelin was less than 3, which does not seem improbably low. We conclude that the currents through the myelin are adequately explained without postulating an 'active' process there as well as at the nodes.

All our analysis so far has been made on the assumptions that the longitudinal current in the axis cylinder is equal and opposite in direction to that in the fluid outside the myelin, and that the axis cylinder is a uniform ohmic conductor. These are standard assumptions of the membrane theory, but it will be well to see if there is any reason to doubt them, and, if so, whether our analysis is affected.

As regards the first of these assumptions, it is conceivable that some current flows either between a surface membrane of the axis cylinder and the myelin or between the lipoid layers of the myelin itself. If these were merely passive currents, in parallel with either that in the axis cylinder or with that in the external fluid, they would be negligible in magnitude because of the extremely

high resistance of such pathways. On the other hand, such currents might be produced locally by a conduction process taking place entirely within the myelin sheath and axis cylinder. It was in order to test this possibility that we carried out the experiment described in the last part of the 'Results' section. This showed that conduction could be prevented by raising the external resistance. This result agrees with those of Hodgkin (1937 *a*, *b*, 1939) and Tasaki (1939) in indicating that conduction depends on external currents. We conclude that there is no conduction process entirely within the myelin sheath and axis cylinder, and that it is justifiable to assume that the current in the axis cylinder is equal and opposite to that flowing in the external fluid.

As regards the second assumption, the axis cylinder is not uniform since it is markedly constricted at the nodes of Ranvier. The constrictions extend for so short a distance along the fibre, however, that it is justifiable to neglect their effect on resistance. Our analysis might, however, be seriously affected if the axis cylinder is interrupted by transverse membranes across which potential differences can be developed, as suggested by von Muralt (1945). It is difficult to say how this theory would alter our analysis, since it has not yet been given quantitative expression. We are inclined to think that the theory would predict action currents similar qualitatively to those required by the usual theory of a membrane concentric with the axis of the fibre, so that our results do not provide evidence either way concerning the theory.

Node spacing and conduction velocity. Sanders & Whitteridge (1946) found that a large change in node spacing could occur without an appreciable change in conduction velocity. This is generally regarded as evidence against the theory of saltatory conduction. This argument depends on the assumption that a saltatory system will always conduct faster the greater the distance between nodes, as in the simplified case treated by Offner *et al.* (1940). The following examples show that this is by no means necessarily so.

Consider first the case where the conductance and capacitance of the myelin and the conductance of the membrane at a node are negligible. The active node has then to charge the membrane capacity of adjacent nodes through the longitudinal resistances of the axoplasm and the fluid surrounding the fibre.

Let C_n = capacity of membrane at a node,
l = distance between adjacent nodes.
V_m, V_0, r_1, r_2 have the same significance as in the preceding section.

Suppose that as soon as V_m at a node reaches a fixed value V_c it drops instantaneously to a lower value V_a and stays at that value for a time long enough for subsequent changes to have no appreciable effect on the conduction velocity. If, then, the equations and boundary conditions defining the time course of $(V_m - V_0)/(V_a - V_0)$ are expressed in terms of a time variable $T = t/(r_1 + r_2)\, lC_n$, they do not contain r_1, r_2, l or C_n. The interval between the

moments at which successive nodes reach V_c is therefore constant in units of T, whatever values those parameters may have. It will depend only on $(V_a - V_0)/(V_c - V_0)$, so that we can say

$$\Delta T = f\left\{\frac{V_a - V_0}{V_c - V_0}\right\}.$$

Hence

$$\Delta t = (r_1 + r_2)\, l C_n f\left\{\frac{V_a - V_0}{V_c - V_0}\right\},$$

and the velocity is

$$l/\Delta t = \frac{1}{(r_1 + r_2)\, C_n f\left\{\dfrac{V_a - V_0}{V_c - V_0}\right\}},$$

which is independent of node spacing.

The simplifications on which this result depends are certainly too drastic both for very short and for very long internodes. In the former case, our assumptions would require the currents to become very large. Real conditions will therefore be represented better if we imagine that the source of potential which charges each node from V_c to V_a has an internal resistance r. The dimensional argument will now apply only so long as $r/(r_1 + r_2)\, l$ is constant, and then, for given values of V_c and V_a,

$$\Delta T = \phi\left\{\frac{r}{(r_1 + r_2)\, l}\right\}.$$

Clearly an increase in r will delay the charging of the next node, so that $\phi\left\{\dfrac{r}{(r_1 + r_2)\, l}\right\}$ increases as $r/(r_1 + r_2)\, l$ increases. The velocity is

$$\frac{l}{\Delta t} = \frac{l}{(r_1 + r_2)\, l\, C_n \phi\left\{\dfrac{r}{(r_1 + r_2)\, l}\right\}} = \frac{1}{(r_1 + r_2)\, C_n \phi\left\{\dfrac{r}{(r_1 + r_2)\, l}\right\}},$$

which increases with l, approaching for large l the constant value it would have if $r = 0$.

When the internodes are long, the distributed capacity of the myelin must become important, and in the limit the capacity of the nodes will be negligible in comparison with it. In that case,

$$1/(r_1 + r_2) \,.\, \partial^2 V_m/\partial x^2 = C_m \partial V_m/\partial t,$$

where C_m = capacity of myelin sheath per unit length of the fibre. If we now define

$$X = x/l \quad \text{and} \quad T = t/(r_1 + r_2)\, l^2 C_m,$$

this equation becomes

$$\partial^2 V_m/\partial X^2 = \partial V_m/\partial T,$$

and the boundary conditions do not involve r_1, r_2, l or C_m. Hence the time per internodal distance is constant in units of T, and proportional to $(r_1 + r_2)\, l^2 C_m$

in units of t. The conduction velocity is therefore proportional to $1/(r_1+r_2)\,lC_m$, which actually decreases as node spacing increases. At still greater internodal spacing, the conductance, both of the myelin and of the nodes, may become important, reducing velocity still further and eventually causing a block.

On this set of assumptions, therefore, one would expect that the conduction velocity will have a maximum at a particular node spacing, and that it might be a very flat maximum. Natural selection will probably have made the normal spacing fall near this optimum, so that considerable deviations from the normal spacing would cause only small changes in velocity.

Sanders & Whitteridge compared the velocity in normal fibres with that in fibres with considerably reduced internodal lengths. We should expect the difference in velocity to be least if the normal spacing were somewhat above, and the reduced spacing below, the value which would give maximum velocity. There is, in fact, some evidence to suggest that, at least in frog and toad fibres, the normal spacing is above the value for maximum velocity. Thus, Tasaki & Takeuchi (1941) recorded 'mononodal' action currents from a node both when only one and when both of the neighbouring nodes were narcotized. The records were practically identical, suggesting that the internal resistance of the active node, the factor which makes conduction velocity increase with node spacing, is negligible. Further, our membrane current records (Fig. 12) show that the capacity of the myelin in an internode is greater than the capacity of the nodal membrane, so that the conditions approach those in which it was shown above that velocity is inversely proportional to node spacing.

SUMMARY

1. The longitudinal current which flows in the external fluid when an impulse passes was recorded at various positions along a fibre isolated from the sciatic nerve of *Rana esculenta* or *R. temporaria*.

2. The conduction time of the longitudinal action current was found to be practically constant in each internode, and to increase stepwise as each node of Ranvier was passed.

3. The amplitude of the first phase of the longitudinal current was found to fall steadily from the proximal to the distal end of each internode, and to increase suddenly as each node was passed.

4. The current crossing the myelin sheath during the impulse could be explained as a passive current due to the potential change acting on a resistance and capacity in parallel.

5. At each node, positive current began to enter the axis cylinder before the potential change had reached its maximum. This relation is impossible in a system of resistances and capacities and is shown to be a necessary characteristic of the points which maintain decrementless conduction in a cable-like structure. It is concluded that the process which gives rise to the action

potential takes place at the nodes of Ranvier, confirming the theory of saltatory conduction.

6. Conduction was blocked reversibly by increasing the external resistance between two adjacent nodes. It is concluded that the action potential at each node excites the next node by current flowing forward in the axis cylinder and back in the fluid outside the myelin sheath.

7. The finding of Sanders & Whitteridge that a large decrease in node spacing can occur without a drop in conduction velocity is shown not to conflict with the theory of saltatory conduction.

This work was made possible by a grant from the Rockefeller Foundation, to whom we wish to express our gratitude. We are also deeply indebted to Mr A. L. Hodgkin for much valuable discussion and criticism, and for the loan of apparatus.

REFERENCES

Bielschowsky, M. (1928). *Handbuch der Mikroskopischen Anatomie des Menschen*, **4**, 98 et seq. Berlin: Springer.
Cole, K. S. & Baker, R. F. (1941). *J. gen. Physiol.* **24**, 535.
Cole, K. S. & Curtis, H. J. (1939). *J. gen. Physiol.* **22**, 649.
Cole, K. S. & Hodgkin, A. L. (1939). *J. gen. Physiol.* **22**, 671.
Erlanger, J. & Blair, E. A. (1934). *Amer. J. Physiol.* **110**, 287.
Erlanger, J. & Blair, E. A. (1938). *Amer. J. Physiol.* **124**, 341.
Erlanger, J. & Gasser, H. S. (1937). *Electrical Signs of Nervous Activity*, p. 29. Philadelphia: University of Pennsylvania Press.
Grundfest, H. (1947). *Ann. Rev. Physiol.* **9**, 488.
Hodgkin, A. L. (1937*a*). *J. Physiol.* **90**, 183.
Hodgkin, A. L. (1937*b*). *J. Physiol.* **90**, 211.
Hodgkin, A. L. (1939). *J. Physiol.* **94**, 560.
Hodgkin, A. L. (1947). *J. Physiol.* **106**, 305.
Hodgkin, A. L. & Huxley, A. F. (1945). *J. Physiol.* **104**, 176.
Hodgkin, A. L. & Rushton, W. A. H. (1946). *Proc. Roy. Soc.* B, **133**, 444.
Huxley, A. F. & Stämpfli, R. (1948). *Helv. physiol. pharmacol. Acta*, **6**, C 22.
Kato, G. (1934). *Microphysiology of Nerve.* Tokyo: Maruzen.
Kato, G. (1936). *Cold Spr. Harb. Sym. quant. Biol.* **4**, 202.
Kubo, M., Ono, S. & Toyoda, H. (1934), *Jap. J. med. Sci. Biophys.* **3**, 213.
Lillie, R. S. (1925). *J. gen. Physiol.* **7**, 473.
Maximow, A. A. & Bloom, W. (1942). *Textbook of Histology*, 4th ed. p. 196. Philadelphia: Saunders.
von Muralt, A. (1945). *Die Signalübermittlung im Nerven*, pp. 229 et seq. Basle: Birkhäuser.
Offner, F., Weinberg, A. & Young, G. (1940). *Bull. math. Biophys.* **2**, 89.
Pfaffmann, C. (1940). *J. cell. comp. Physiol.* **16**, 407.
Rushton, W. A. H. (1937). *Proc. Roy. Soc.* B, **124**, 210.
Sanders, F. K. & Whitteridge, D. (1946). *J. Physiol.* **105**, 152.
Stämpfli, R. (1946). *Helv. physiol. pharmacol. Acta*, **4**, 411.
Takeuchi, T. & Tasaki, I. (1942). *Pflüg. Arch. ges. Physiol.* **246**, 32.
Tasaki, I. (1939). *Amer. J. Physiol.* **127**, 211.
Tasaki, I. (1940). *Pflüg. Arch. ges. Physiol.* **244**, 125.
Tasaki, I., Amikura, H. & Mizushima, S. (1936). *Jap. J. med. Sci. Biophys.* **4**, 53.
Tasaki, I. & Takeuchi, T. (1941). *Pflüg. Arch. ges. Physiol.* **244**, 696.
Tasaki, I. & Takeuchi, T. (1942). *Pflüg. Arch. ges. Physiol.* **245**, 764.

J. Physiol. (1949) 108, 340–344 612.766.1:612.172.4

THE EFFECT OF POSTURAL AND EXERCISE COMPONENTS ON THE HEART RATE DURING A BRIEF STEP TEST

By J. A. C. KNOX

From the Department of Physiology, King's College, London

(*Received* 26 *June* 1948)

The starting-point of this investigation was an apparent anomaly in the curve of heart rate during a brief step test. The subjects were seated, and at a given signal they stood up, stepped five times up and down two steps at a constant rate and immediately returned to the sitting posture. The heart beats were electrically recorded throughout exercise and were counted in 5 sec. periods to the nearest tenth of a beat. The average time for the whole exercise was just over 20 sec. and it was found that the mean curve of heart rate for seventy-five male subjects showed a distinct drop in rate during the last 5 sec. of exercise (Knox, 1940).

It was very unlikely that a steady state had been reached in such a short time; and other workers, using comparable exercises but less delicate methods of counting, have generally found that the heart rate increases steadily until the end of exercise. It seemed possible that in a short light exercise of the present type, where the heart rate was counted accurately over very small periods, the effects of posture might appear in the record and be a complicating factor. The exercise was begun from the sitting posture and standing erect might be expected to augment the heart rate during the first part of the exercise. This postural increase might pass off as circulatory adjustments were made, independent of the actual stepping, thus accounting for the decrease in rate during the last 5 sec. of exercise.

This hypothesis was tested on a series of seven male students who were accustomed to the apparatus and in whom the psychological increase of heart rate was minimal. This presumably accounts for their low maximum rates.

METHOD

First, the postural component alone was analysed. The subjects sat down and rested until the heart rate was steady, then they stood up and remained standing quietly. Next, the effect of posture plus exercise was recorded, the subjects performing the standard exercise of five ascents of two steps beginning and ending in the sitting posture. Finally the subjects stood quietly for 5 min. and

then repeated the step test beginning and ending in the standing posture. Between each test the subjects rested for 15 min. The heart beats were electrically recorded on a smoked drum throughout the three tests and were counted over successive 5 sec. intervals to the nearest tenth of a beat.

RESULTS

Graphs of the mean heart rates in 5 sec. intervals during the three tests are shown in Fig. 1. It will be seen that the curve for the sitting-exercise-sitting test (curve B) shows the typical decrease in rate during the last 5 sec. of exercise,

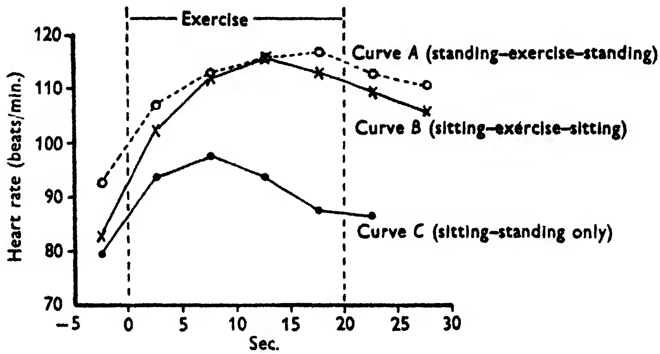


Fig. 1. Graphs of mean heart rates in 5 sec. periods during the step test, and the effect of posture alone (seven male subjects). Curve A: exercise performed beginning and ending in the standing posture; curve B: exercise performed beginning and ending in the sitting posture; curve C: mean heart rates when the subjects changed from the sitting to the standing posture.

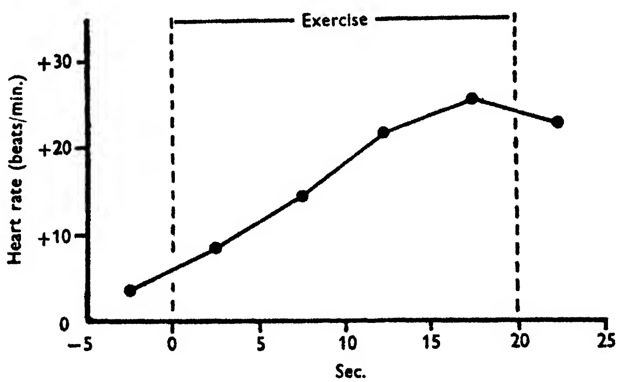


Fig. 2. Graph of mean heart rate in successive 5 sec. periods obtained by subtracting curve C from curve B (Fig. 1). This gives the rise of heart rate due to the exercise component alone (seven male subjects).

whereas the standing-exercise-standing curve (curve A) continues to rise until the end of exercise. The sitting-standing postural curve (curve C) shows an increase to a maximum followed by a decrease to a resting level about seven beats per minute higher than the sitting resting rate.

As might be expected from the postural hypothesis the curve of acceleration of the heart rate during the first 10 sec. of exercise is steeper in the sitting-exercise-sitting than in the standing-exercise-standing test. It would thus appear that the postural increase and the exercise increase are to a certain extent additive. If this is so, it should be possible to construct a curve for the exercise component alone by subtracting the heart rates in each 5 sec. period of the sitting-standing test from those in the corresponding periods of the sitting-standing-exercise test, i.e. by subtracting curve *C* from curve *B*. If the components are additive one would expect the resultant curve to rise steadily to a maximum at the end of exercise as in curve *A* (standing-exercise-standing). The resultant curve is shown in Fig. 2 and follows the general trend expected.

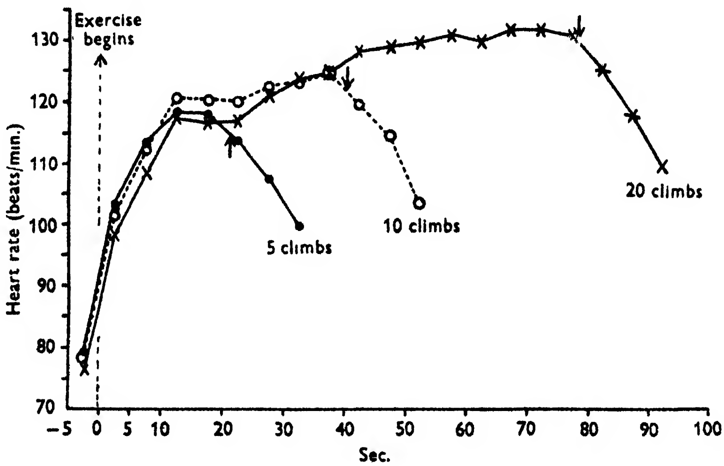


Fig. 3. Graphs of mean heart rates in 5 sec. intervals showing the effect of varying the duration of the exercise. The exercise consisted of 5, 10 and 20 ascents of two steps at the standard rate of 96 steps per minute beginning and ending in the sitting posture. The small arrows indicate the end of exercise for each curve. The curves for 10 and 20 climbs show a plateau due to the withdrawal of the dynamic postural component (thirteen male subjects).

In addition it is to be expected that if the exercise were continued beyond the standard 20 sec. the heart rate would increase again after the drop due to the falling off in the postural component. This is clearly demonstrated in Fig. 3, which shows graphs of the mean heart rates of thirteen subjects who performed successively 5, 10 and 20 ascents of the two steps at the standard speed, beginning and ending in the sitting posture. The curves for 10 and 20 ascents show a typical plateau due to the withdrawal of the dynamic postural component. This begins about 12·5 sec. after the commencement of exercise and lasts about 10 sec., thus showing the same time relations as the postural increase curve in Fig. 1 (curve *C*). This effect of the postural component can only be

demonstrated when the exercise is very light, as with more severe exercise the postural component is overwhelmed by the steadily increasing exercise component.

DISCUSSION

The postural component is itself made up of two factors, the dynamic muscular movement of standing up and the steady gravitational factor when erect. The first would account for the primary rise and fall of heart rate seen in Fig. 1, curve *C*; and the second would be responsible for the maintained increase in rate. It is, of course, the passing off of the dynamic factor which is responsible for the fall in heart rate during the brief standard exercise employed here.

In the present series the standing resting rate finally became steady at about seven beats per minute above the sitting resting rate when counting was continued beyond the 25 sec. shown in Fig. 1, curve *C*. This is in general agreement with the average results given by previous workers for the sitting-standing increase. Vierordt (1906) quoted nine beats per minute as an average figure, while Schneider (1916) gave ten beats per minute for men, and Turner (1927*a*), thirteen beats per minute for women. The whole question of postural increase in rate was exhaustively studied by MacWilliam (1933), who found wide variations in the sitting-standing increase, but eight beats per minute was a usual figure for his subjects. His final conclusion was that 'the vertical position of the thighs in standing is the essential condition of the quicker pulse rate'. In his experiments the rate does not appear to have been counted in intervals smaller than half a minute, however, and the brief initial rise and subsequent fall shown in Fig. 1 were thus not considered.

Helmreich (1923) seems to have been the first to recognize that even the simple act of standing erect involves two distinct components which may affect the heart rate. These caused 'a dynamic rise corresponding to the increased use of oxygen seen in exercise, and a static rise corresponding to the changed relationship of the body to gravity'. As was mentioned above, these components are clearly seen in the primary rise and subsequent plateau of the sitting-standing curve in Fig. 1. The existence of such a dual mechanism is also implicit in the results of Turner (1927*b*, 1929) though no quantitative data were given bearing on this particular point. In 1930, however, Turner, Newton & Haynes investigated the postural component alone by having the subject strapped to a tilting table so that no exercise was involved in the change of posture. They found that the delicacy of the circulatory reaction to gravity was such that a tilt of only 15° produced a definite effect on the heart rate. This technique was extended to include exercise by Asmussen, Christensen & Nielsen (1939). Their subject was placed on a tilting bed and performed rhythmic arm movements against elastic resistance. The heart rate increased on tilting into the head-up position and if this tilt took place during the arm work the postural increase was added to the exercise increase. The postural

increase was prevented or reversed on clamping the legs. The authors ascribed the postural effect to diminished pressure in the central veins.

Thus in the previous work the separation of the postural and exercise components was carried out under artificial conditions, whereas the present experiments show that they have a distinct and additive effect on the heart rate during a natural mixed exercise. They also provide an example of the delicacy with which the cardiac centres respond to afferent impulses, and of the fact that the heart rate at any given moment is a resultant of the effects of all these afferents.

SUMMARY

1. When a brief step test is performed, beginning and ending in the sitting posture, there are two factors which may affect the heart rate: (*a*) the postural component, due to the change of posture from sitting to standing; and (*b*) the exercise component, due to the actual stepping.

2. By recording the heart beats electrically during exercise it is shown that each of these factors does influence the heart rate and that their effects can be separated but are additive in the mixed exercise. A fall in heart rate observed during the step test is due to the partial regression of the postural component.

REFERENCES

Asmussen, E., Christensen, E. H. & Nielsen, M. (1939). *Skand. Arch. Physiol.* **81**, 225.
Helmreich, E. (1923). *Z. ges. exp. Med.* **36**, 226.
Knox, J. A. C. (1940). *Brit. Heart J.* **2**, 289.
MacWilliam, J. A. (1933). *Quart. J. exp. Physiol.* **23**, 1.
Schneider, E. C. (1916). *Amer. J. Physiol.* **40**, 380.
Turner, A. H. (1927*a*). *Amer. J. Physiol.* **80**, 601.
Turner, A. H. (1927*b*). *Amer. J. Physiol.* **81**, 197.
Turner, A. H. (1929). *Amer. J. Physiol.* **87**, 667.
Turner, A. H., Newton, M. I. & Haynes, F. W. (1930). *Amer. J. Physiol.* **94**, 507.
Vierordt, H. (1906). *Anatomische, Physiologische und Physikalische Daten und Tabellen.* Jena: Fischer.

J. Physiol. (1949) 108, 345–352 612.492.8:612.12

THE EFFECT OF THE POSTERIOR PITUITARY HORMONES ON THE INORGANIC PHOSPHORUS AND SUGAR OF THE BLOOD

By A. M. FRASER

From the Department of Pharmacology, McGill University, Montreal

(*Received* 1 *July* 1948)

Stehle & Bourne (1925) found that the urinary excretion of total phosphorus was increased during pituitary diuresis in dogs; Bolliger & Hartman (1925) noted an increase in blood inorganic phosphates in dogs following the intravenous injections of pituitary extract; Gollwitzer-Meier (1926) obtained the same results using rabbits. All used whole pituitary extract in quite large doses, ranging from 10 units upward. Later Geiling, DeLawder & Rosenfeld (1931), studying the effect of intravenous injections of 1 ml. of pitocin and 0·5 ml. pitressin after insulin in dogs, found that pitressin decreased the fall in inorganic phosphate of the blood caused by insulin, while pitocin had no effect.

Fraser (1942) reported that small doses of highly purified oxytocic hormone decreased the urinary excretion of phosphorus and suggested that the action may be of physiological significance, since it was elicited by doses as small as 0·0001 unit or 0·0004 μg./kg. of dog. The results also indicated that the opposite effect, observed in previous work with large doses of whole pituitary extract, was probably due to the pressor hormone.

It appears likely that the influence of posterior pituitary extracts on phosphorus excretion is associated with their effects on carbohydrate metabolism. However, in view of the well-known diuretic and antidiuretic actions of the pressor hormone, and the less well-known diuretic action of the oxytocic hormone (Fraser, 1937; Bundschuh & Kuschinsky, 1939; Kuschinsky & Bundschuh, 1939*a*, *b*; Fraser, 1942), the possibility that the effects of these hormones on phosphorus excretion are due to, or essentially associated with, their action on water and chloride excretion, must be considered.

The purpose of the present work was to study the effects of highly purified preparations of the oxytocic and pressor hormones on the inorganic phosphorus of the blood, and to correlate any effects with concomitant changes in blood-sugar and phosphorus excretion, in the hope of throwing some light on the mechanisms involved. The unexpected findings on blood sugar led to some experiments dealing solely with these changes.

METHOD

Preparations. Postlobin-V and postlobin-O, Stehle's (1933) highly purified preparations of the pressor and oxytocic hormones respectively, were used in this work. The extracts employed were prepared in this laboratory by a modified method (Stehle & Fraser, 1935). The postlobin-V contained 200 pressor units and 10 oxytocic units/mg., and the postlobin-O contained 250 oxytocic units and 5 pressor units/mg. Therefore the administration of 1 unit of postlobin-V was necessarily accompanied by 0·05 oxytocic unit; similarly, 1 oxytocic unit of postlobin-O was contaminated with 0·02 pressor unit. Oxytocic values are based on assays carried out on the guinea-pig uterus in magnesium-free Ringer-Locke.

Chemical methods. Blood sugar was determined by the Andes & Northup (1939) adaptation of the Folin-Wu method. Plasma inorganic phosphorus was determined by the method of Benedict (1924) adapted to the photoelectric colorimeter. Total phosphorus determinations of the urine were made by evaporation to dryness, treatment with sulphuric and nitric acids, and hydrogen peroxide, followed by precipitation and weighing according to Pregl (1930).

Physiological technique. Dogs were used in all experiments. They were fed daily at 4 p.m. with a constant meat diet. On the day prior to the experiment they were giv n water by stomach tube at 5 p.m., in an amount equivalent to about 2·5 % of their body weight; remaining food or water was removed from their cages at this time. The experiment usually began about 10 a.m., and 2 days were always allowed to elapse between experiments on any one animal. Controls were carried out with most experiments, and the substance injected was chosen so that the rotation, postlobin-O, saline, postlobin-V, was maintained in successive experiments, to minimize errors produced by possible changes in the sensitivity of the animal. These injections were made in a volume of 0·05 ml./kg.

In the studies on phosphorus changes, a bladder fistula bitch, weighing 18 kg., was used. This dog was trained to lie on a table, and the urine was collected in graduated cylinders as it dropped from the ureteral orifices. Urine collections were made during each 15 min. interval, and venous blood samples were taken every 15 min. Thirty minutes after the beginning of the experiment an intravenous injection of postlobin-O, postlobin-V or physiological saline was made. The experiments were terminated after 105 min.

In the experiments concerned solely with blood-sugar changes two normal dogs were used, a black female weighing 11·5 kg. and a white male weighing 13·5 kg. Blood samples were taken at 0, 5, 10, 15, 20 and 35 min. after the beginning of these experiments.

RESULTS

The results concerned with phosphorus are summarized as averages in Tables 1 and 2. The dose of 0·025 oxytocic unit of postlobin-O/kg. was chosen because it gave a significant, yet submaximal hyperglycaemia, as well as a consistent decrease in plasma inorganic phosphate. The dose of 0·5 pressor unit of postlobin-V/kg. was chosen because it contained the same amount of oxytocic activity (as contaminant) as did the dose of postlobin-O, namely, 0·025 unit/kg. This dose of postlobin-V was not large enough to cause circulatory collapse, but it produced visible pallor of the mucous membranes for 45 min. The results in Table 1 indicate a drop in plasma inorganic phosphate following the oxytocic preparation, whereas there is quite as definite a rise following the injection of a dose of the pressor preparation, which contains equivalent oxytocic activity as contaminant. Evidently the action of the pressor hormone has not only masked the phosphorus-lowering action of the oxytocic hormone, but has

TABLE 1. Blood sugar and inorganic phosphate in a dog after equal oxytocic doses of pressor and oxytocic extracts

Minutes from start ...	No. of exps.	0		15		30		45		60		75		90		105	
		G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P
Controls	4	100	3·82	104	3·62	105	3·49	105	3·49	103	3·51	104	3·52	104	3·47	104	3·42
0·025 oxytocic unit P-O	5	102	3·75	103	3·54	101	3·28	138	2·75	114	2·52	108	2·48	106	2·56	103	2·53
0·5 pressor unit P-V (0·025 oxytocic unit)	5	101	3·34	99	3·22	97	3·04	188	3·34	129	4·14	114	3·75	103	3·50	104	3·37

G = glucose (mg. %). P = inorganic phosphorus (mg. %). P-O = postlobin-O (dose/kg.).
P-V = postlobin-V (dose/kg.). Injections made at 30 min.

TABLE 2. Total urine phosphorus and volume in a dog after equal oxytocic doses of pressor and oxytocic extracts

Interval (min. from start) ...	No. of exps.	0–15		15–30		30–45		45–60		60–75		75–90		90–105	
		P	V	P	V	P	V	P	V	P	V	P	V	P	V
Controls	4	2·86	3·4	2·32	3·8	1·75	2·9	1·62	2·9	1·53	2·8	1·26	2·5	1·21	2·2
0·025 oxytocic unit P-O	5	6·10	4·9	3·59	4·6	4·60	3·6	1·70	4·0	0·65	3·6	0·29	3·2	0·50	2·9
0·5 pressor unit P-V (0·025 oxytocic unit)	5	2·37	3·6	1·58	3·4	1·61	1·7	9·91	2·5	5·19	6·1	9·02	9·5	6·70	7·9

P = total phosphorus (mg.). V = urine volume (ml.). P-O = postlobin-O (dose/kg.).
P-V = postlobin-V (dose/kg.). Injections made at 30 min.

actually raised the plasma phosphorus above normal. These changes occurred consistently in all experiments. In all experiments, including controls, the plasma phosphorus decreased somewhat during the first 30 min.; this is possibly associated with the activity and apprehension of the dog during this early part of the experiment.

The results on urinary phosphorus and water excretion are not new, but merely confirm conclusions drawn from the earlier work (Fraser, 1942) that the pressor and oxytocic hormones raise and lower phosphorus excretion respectively.

The most interesting feature of the results, in Table 1, on blood sugar, is the much stronger action of the pressor preparations despite the fact that in both sets of experiments equal doses of oxytocic activity were administered. This stronger action of the pressor preparation was a consistent one; that is, in all five experiments the maximal rise was greater than the maximal rise in any of the five experiments using the oxytocic preparation. This finding is contrary to that of Holman & Ellsworth (1935), who concluded that the oxytocic hormone is responsible for the hyperglycaemic activity of posterior pituitary extracts in dogs. Additional experiments, in which only blood-sugar changes were studied, were therefore carried out in order to check our unexpected finding. Since the results in Table 1 had shown that the hyperglycaemic responses had almost disappeared in 30 min., blood samples were taken over a shorter period, and more frequently. The doses employed were the same as those used in the original series of Table 1. The results are shown in Table 3. They confirm those in Table 1, in that the pressor preparation has a greater hyperglycaemic activity than the oxytocic, even though equivalent doses, in terms of oxytocic activity, were given. It is worth noting, however, that the hyperglycaemic responses are approximately equal 5 min. after the injections, the large differences appearing at 10 and 15 min. following the administration of the extracts.

TABLE 3. Blood sugar (mg. %) after equal oxytocic doses of pressor and oxytocic extracts

No. of exps.	Minutes from start ...	0	5	10	15	20	35
	White male dog						
5	0·025 oxytocic unit P-O	101	104	137	141	127	110
5	Controls	103	104	105	107	104	101
5	0·5 pressor unit P-V (0·025 oxytocic unit)	103	102	134	168	140	108
	Black female dog						
5	0·025 oxytocic unit P-O	99	100	120	124	112	104
5	Controls	99	102	101	99	99	99
5	0·5 pressor unit P-V (0·025 oxytocic unit)	92	95	123	150	139	103

P-O = postlobin-O (dose/kg.).
P-V = postlobin-V (dose/kg.).
Injections were made at 5 min.

The hyperglycaemic activity of the above doses of oxytocic extract was compared with that of one quarter the dose of pressor extract used above, in an attempt to match their actions. The results are shown in Table 4. Maximal hyperglycaemic responses occurred 5 min. after the injections of the oxytocic extract, but not until 10 min. following the injections of the pressor extract. Further, the maximal hyperglycaemic response to this small dose of pressor extract was greater than the response to the oxytocic extract in one dog, and approximately equal to it in the other. Thus the degree of hyperglycaemic response to the pressor extract is at least four times as great as can be accounted for by its oxytocic contamination. The quality of the response also differs from that of the oxytocic extract, and the relative sensitivity to the two extracts differs in the two dogs.

TABLE 4. Comparison of blood sugar (mg. %) in dogs after oxytocic extract with that after a dose of pressor extract containing 25% as much oxytocic activity

No. of exps.	Minutes from start ...	0	5	10	15	20	35
	Black female dog						
5	0·025 oxytocic unit P-O	97	97	118	119	110	100
5	0·125 pressor unit P-V (0·00625 oxytocic unit)	101	97	106	127	115	100
	White male dog						
5	0·025 oxytocic unit P-O	101	103	140	141	132	108
	0·125 pressor unit P-V (0·00625 oxytocic unit)	105	105	117	139	128	111

P-O = postlobin-O (dose/kg.).
P-V = postlobin-V (dose/kg.).
Injections made at 5 min.

DISCUSSION

The hyperglycaemic action of the oxytocic hormone is evidently associated with its ability to decrease urinary and blood phosphorus.

Apparently adrenaline plays no role in the hyperglycaemic action of pituitary extract. Clark (1928), and Thaddea & Waly (1933) found that blood sugar still rose after adrenalectomy, while Clark (1927), Geiling & Britton (1927) and others also observed pituitary hyperglycaemia after ergotamine. As far as is known, pituitary extract hyperglycaemia is due to a direct action on the liver resulting in the liberation of glucose from glycogen, for Clark (1928) found no hyperglycaemia with pituitary extract when the liver was isolated from the circulation. Many others reached Clark's conclusion by various types of experiments. Although the work referred to above was carried out with whole pituitary extract, it is reasonable to conclude that in the present experiments the hyperglycaemic action of the oxytocic hormone is also on the liver, especially since the effect of this hormone on blood phosphorus is consistent with this view, as will be evident below.

It has been well established that the administration of glucose, insulin or adrenaline to normal animals causes a decrease in the inorganic phosphates of the blood (Cori & Cori, 1931, 1932; Bolliger & Hartman, 1925; Harrop & Benedict, 1924), and that these decreases are due to insulin (Soskin, Levine & Hechter, 1941). One may tentatively assume, therefore, that the hyperglycaemia, produced by the action of the oxytocic hormone on the liver, stimulates the secretion of insulin, and so decreases the blood phosphorus in the same way as does the administration of insulin, glucose or adrenaline.

The present results indicate that the pressor hormone itself possesses a hyperglycaemic action in the dog. The delay in the onset of the hyperglycaemia following the pressor extract, as compared with the more immediate response to the oxytocic extract (Table 4), and the difference in the relative sensitivity of the dogs to the two extracts, strongly suggest that the mechanisms involved are different. As pointed out above, when equal oxytocic doses of the two extracts are administered the immediate hyperglycaemic responses are approximately equal (Table 3); the greater response to the pressor extract appears only 10 and 15 min. following administration. All this suggests that a hyperglycaemic action of the pressor hormone is superimposed upon that of the oxytocic hormone, when pressor extracts are given. The rise in inorganic phosphate of the plasma, and the increased excretion of phosphorus caused by the pressor hormone, do not support a view that the pressor hormone simply increases the liberation of glucose into the blood.

How does the pressor hormone cause these increases in sugar and phosphorus? The smaller dose of pressor extract used in this work, viz. 0·125 unit/kg., produced very obvious pallor of the mucous membranes, and a state of tissue anoxia was probably present. Anoxic anoxia is known to produce (Van Liere, 1942) increased H-ion concentration and decreased alkali reserve of the blood, increased blood sugar and increased blood lactic acid. Asphyxia increases plasma potassium and potassium excretion, but the data on the effect of anoxia on potassium are conflicting. Gravity shock, in which there is a state of stagnant anoxia, leads to similar blood changes (Cole, Allison, Murray, Boyden, Anderson & Leathem, 1944). In this condition the blood of rabbits was found to have increased potassium, increased lactate, increased inorganic phosphate, increased H-ion concentration and decreased alkali reserve; in fasting animals the blood sugar dropped, but in fed animals it rose markedly. All these blood changes were ascribed to the stagnant tissue anoxia, and the consequent interference with the aerobic phase of carbohydrate metabolism. There is a striking similarity between these blood changes observed in states of anoxia and those which follow administration of pituitary extract. Pituitary extract decreases alkali reserve and increases H-ion concentration (Gollwitzer-Meier, 1926), it increases potassium excretion (Stehle, 1927) and increases blood lactic acid (Nitescu & Munteanu, 1931; Bischoff & Long, 1931–2), and there is evidence

that oxygen consumption decreases (Geiling & DeLawder, 1932). It is therefore suggested that these changes, and the phosphorus and sugar increases observed in the present experiments, are secondary to the intense peripheral vasoconstriction, and the consequent tissue anoxia, which follows administration of pressor hormone.

Another possible mechanism by which the pressor hormone could raise the sugar and phosphorus of the blood should be mentioned. It is well known that a rise in plasma inorganic phosphate, increased phosphorus excretion, as well as a rise in blood sugar, follow pancreatectomy. It is therefore possible that the pressor hormone produces the same phenomena by inhibition of insulin secretion. Such an action could also be secondary to a circulatory effect.

Though the pressor hormone possesses some hyperglycaemic activity, the oxytocic hormone must be regarded as largely responsible for the hyperglycaemic effect of posterior pituitary extract in the dog, in agreement with Holman & Ellsworth (1935). That this action of the pressor hormone escaped the notice of these workers may be due to relative insensitivity of their animals to it. A species variation in sensitivity to the hyperglycaemic actions of both hormones is well known. For example, in man, the hyperglycaemic effects of posterior pituitary extract have been attributed to the pressor hormone (Gavrila & Mihaileanu, 1930; Elmer & Scheps, 1930; Schroeder, 1933; Thaddea, 1933). In Schroeder's work on man (1933) the maximal rises in blood sugar after 10 units pitressin are about the same as those following a similar dose (0·125 unit/kg.) in the present experiments in dogs. Possibly the only difference, in sensitivity, between these two species, is with respect to the oxytocic hormone. It should be emphasized that, whatever the mechanism, the hyperglycaemic action of the pressor hormone is probably not of physiological significance, since the doses required to elicit effects are so much larger than those which affect urine secretion.

The preparations used here were assayed, as stated above, on guinea-pig uterus in magnesium-free Ringer-Locke. It has been shown that in the presence of magnesium the pressor hormone exerts an oxytocic action (Fraser, 1939), so that now oxytocic assays are frequently carried out in the fluid of Van Dyke & Hastings (1928) which contains magnesium in the concentration which exists in the blood plasma. When this method is used, the oxytocic activity of the pressor extract employed here is increased four times (Fraser, 1939). It is necessary, therefore, to use a magnesium-free fluid to estimate oxytocic hormone, rather than total physiological oxytocic activity. Even in a fluid which is presumably magnesium-free, it is possible that the pressor hormone exerts oxytocic action, due to traces of magnesium or for other reasons. Thus, if our values for the oxytocic contaminations are incorrect, they are too high. If so, the conclusions regarding the hyperglycaemic activity of the pressor hormone are only strengthened.

CONCLUSIONS

1. The oxytocic and pressor hormones of the pituitary gland decrease and increase, respectively, the inorganic phosphate of the plasma in the dog.

2. The hyperglycaemic activity of the pressor preparation used cannot be accounted for by oxytocic contamination, and therefore a hyperglycaemic action in the dog must be attributed to the pressor hormone, in disagreement with previous workers.

3. It appears very probable that the decrease in plasma phosphate following administration of oxytocic hormone is due to increased secretion of insulin, which is caused, in turn, by the hyperglycaemia.

4. It is suggested that the increases of blood phosphate and sugar brought about by pressor hormone are due to tissue anoxia which results from vasoconstriction, or to inhibition of insulin secretion, or to both.

5. The relative sensitivities to the hyperglycaemic properties of the two hormones may show a species variation.

REFERENCES

Andes, J. E. & Northup, D. W. (1939). *J. Lab. clin. Med.* **24**, 530.
Benedict, E. M. (1924). *J. biol. Chem.* **61**, 63.
Bischoff, F. & Long, M. L. (1931–32). *Amer. J. Physiol.* **99**, 253.
Bolliger, A. & Hartman, F. W. (1925). *J. Physiol.* **60**, 229.
Bundschuh, H. E. & Kuschinsky, G. (1939). *Klin. Wschr.* **18**, 251.
Clark, G. A. (1927). *J. Physiol.* **62**, viii.
Clark, G. A. (1928). *J. Physiol.* **64**, 324.
Cole, W. H., Allison, J. B., Murray, T. S., Boyden, A.A., Anderson, J. A., and Leathem, J. H. (1944). *Amer. J. Physiol.* **141**, 165.
Cori, C. F. & Cori, G. T. (1931). *Physiol. Rev.* **11**, 143.
Cori, C. F. & Cori, G. T. (1932). *J. biol. Chem.* **94**, 581.
Elmer, A. W. & Scheps, M. (1930). *Klin. Wschr.* 2439.
Fraser, A. M. (1937). *J. Pharmacol.* **60**, 89.
Fraser, A. M. (1939). *J. Pharmacol.* **66**, 85.
Fraser, A. M. (1942). *J. Physiol.* **101**, 236.
Gavrila, L. & Mihaileanu, G. (1930). *C.R. Soc. Biol., Paris*, **104**, 601.
Geiling, E. M. K. & Britton, S. W. (1927). *Amer. J. Physiol.* **81**, 478.
Geiling, E. M. K. & DeLawder, A. (1932). *Johns Hopkins Hosp. Bull.* **51**, 335.
Geiling, E. M. K., DeLawder, A., & Rosenfeld, M. (1931). *J. Pharmacol.* **42**, 263.
Gollwitzer-Meier, K. (1926). *Z. ges. exp. Med.* **51**, 466.
Harrop, G. A. & Benedict, E. M. (1924). *J. biol. Chem.* **59**, 683.
Holman, D. V. & Ellsworth, H. C. (1935). *J. Pharmacol.* **53**, 377.
Kuschinsky, G. & Bundschuh, H. E. (1939*a*). *Arch. exp. Path. Pharmak.* **192**, 683.
Kuschinsky, G. & Bundschuh, H. E. (1939*b*). *Klin. Wschr.* **18**, 207.
Nitescu, I. I. & Munteanu, N. (1931). *C.R. Soc. Biol., Paris*, **106**, 499.
Pregl, F. (1930). *Quantitative Organic Microanalysis.* Philadelphia.
Schroeder, H. (1933). *Klin. Wschr.* 1766.
Soskin, S., Levine, R. & Hechter, O. (1941). *Amer. J. Physiol.* **134**, 40.
Stehle, R. L. (1927). *Amer. J. Physiol.* **79**, 289.
Stehle, R. L. (1933). *J. biol. Chem.* **102**, 573.
Stehle, R. L. & Bourne, W. (1925). *J. Physiol.* **60**, 229.
Stehle, R. L. & Fraser, A. M. (1935). *J. Pharmacol.* **55**, 136.
Thaddea, S. (1933). *Z. Klin. Med.* **125**, 175.
Thaddea, S. & Waly, A. (1933). *Arch. exp. Path. Pharmak.* **172**, 535.
Van Dyke, H. B. & Hastings, A. B. (1928). *Amer. J. Physiol.* **83**, 563.
Van Liere, E. J. (1942). *Anoxia.* Chicago: University of Chicago.

J. Physiol. (1949) 108, 353–358 612.741:612.222

THE METABOLIC COST OF PASSIVE CYCLING MOVEMENTS

By J. A. SAUNDERS

From the Department of Physiology and Biochemistry, The Medical School, King's College, Newcastle-upon-Tyne

(*Received* 12 *July* 1948)

Benedict & Cathcart (1913), in their comprehensive work on the efficiency of cycling, used various base-lines for determining net efficiency. One of these was the metabolic rate of the subject sitting on the ergometer while the legs were moved passively by means of a motor which rotated the pedals. They were not satisfied that their results represented truly passive movement and placed little emphasis on them.

Lindhard (1915), commenting on this paper, contended that such leg movements, if truly passive, would involve no calorie expenditure at all, although he produced no experimental evidence to support this view. Hill (1922) considered that the metabolism, while the pedals are being driven by a motor, is the most suitable base-line for use in calculating efficiency during cycling, and used Benedict & Cathcart's figures in his estimate of the actual maximum efficiency of human muscle.

Garry & Wishart (1931) are of the opinion that it is impossible to determine the real efficiency of the effector muscles in any human effort, owing to the difficulty of getting an accurate base-line.

As no experimental work has been done on this point since that of Benedict & Cathcart, the observations reported here were made in an attempt to determine the metabolic cost of truly passive cycling movements at two rates of pedal rotation.

APPARATUS AND METHODS

The machine (an 'exercycle') consisted of a triangular bicycle frame with an electric motor which rotated the pedals. The pedals could be left free or engaged at 'slow' or 'fast' speeds. The 'slow' speed was 56 r.p.m. and the 'fast' speed 74 r.p.m. One revolution means a complete (360°) revolution of the pedal axle, both legs undergoing flexion and extension. It was possible to adjust the positions of the saddle and handlebars in relation to each other to suit the comfort of the subjects, but unfortunately the distance from saddle to pedals was fixed and could not be altered. The subjects' feet were kept on the pedals by means of straps. The metabolic rate of the subjects was determined by the indirect method, using a Douglas bag (60 l. capacity) and a mouthpiece

with rubber flap valves, which were tested before use. The gas analysis was done with 10 ml. Haldane apparatus. Analyses were done in triplicate by two individuals. The volumes of gas in the Douglas bags were measured on a 'wet' type flowmeter.

Plan of experiments. The experiments were carried out starting 1½–2 hr. after a light meal, and the routine given below was carried out in all cases, except that the length of time of collecting expired air varied from 4 to 7 min., depending on the ventilation of the subject.

hr.	min.	
0	00	Subject sits on 'exercycle'. Pedals still.
0	30	Mouthpiece in.
0	45	Start collecting expired air: resting sample 'before' (5 min.).
0	50	Mouthpiece out. Rotation of pedals started.
1	05	Mouthpiece in.
1	20	Start collecting expired air: first work sample (5 min.).
1	26	Start collecting expired air: second work sample (5 min.).
1	31	Mouthpiece out. Pedals stopped.
2	01	Mouthpiece in.
2	16	Start collecting expired air: resting sample 'after' (5 min.).
2	21	Mouthpiece out.

The mouthpiece was not retained by the subject during the whole experiment to avoid discomfort and soreness due to salivation, while 15 min. was allowed for adjustment of breathing through the mouth before a sample of expired air was collected (Campbell, Douglas & Hobson, 1921).

Subjects. The subjects were all medical students and their physical measurements are given in Table 1. All subjects appeared normal although subject 5 subsequently developed pulmonary tuberculosis. Particular efforts were made to ensure that the subjects did not have to endure any

TABLE 1. Physical data of subjects

Subject	Age (years)	Sex	Weight nude (kg.)	Height (cm.)
1	25	M.	76·5	185
2	19	F.	58·9	166
3	27	M.	60·9	172
4	19	M.	62·6	184
5	27	M.	57·5	178

avoidable discomfort. The saddle in which they sat was broad and shaped, and although for one subject padding was provided, the other four were comfortable without it. During the rest periods the pedals were fixed in a horizontal position. A reading desk was attached to the handlebars and the subjects were encouraged to read, in order to obviate boredom. Small movements were not prohibited, but any obvious restlessness was noted and its cause removed. All the subjects volunteered the information that they liked acting as subject as they were able to read quietly and with concentration during the experiments. The mouthpiece was supported by a clamp which took the weight of the mouthpiece and attached tubing and could be moved into any position. The Douglas bags were suspended from above.

Computation of calorie expenditure. The computing of the metabolic rate was done in the usual way, the composition of inspired air being deduced from the percentage of nitrogen expired, using Carpenter's tables (1939). The values used for kg.cal./l. oxygen consumed at different R.Q.'s were those of Cathcart & Cuthbertson (1931), which are based on the composition of human body fat.

RESULTS

The total number of observations made on the five subjects was 122, and details are given in Table 2 of the experiments which gave minimum values for the metabolic cost of passive movement, while Table 3 gives the corresponding

maximum and minimum values. The minimum metabolic cost may be due either to a high metabolic rate before and after or to a low value during passive movement. Both factors may play a part. Table 3 shows that the minimum values reported in Table 2 are derived from a low 'work' metabolism and a low or average resting metabolism. The last column in Table 3 gives a measure of the effect of discomfort on the metabolic cost. The high maximum values for subject 1 were probably due to 'keeping time' with the pedal movement.

TABLE 2. Minimum metabolic rate (kg.cal./hr.)

Subject	Rest (*a*)	Pedals driven by motor at 56 r.p.m. (*b*)	Rest (*c*)	Metabolic cost of passive movement $(d) = b - \frac{1}{2}(a+c)$
1	96·3	106·3	89·9	13·2
2	61·0	68·2	54·2	10·6
3	70·5	81·7	62·4	15·2
4	73·0	82·2	65·0	13·2
5	79·9	82·8	71·8	7·0
		74 r.p.m.		
1	96·9	112·6	88·8	19·7
2	61·4	90·4	58·8	30·3
3	80·4	100·1	70·9	24·4
4	79·6	90·7	64·2	18·8
5	79·5	100·2	68·3	26·3

TABLE 3. Minimum and maximum metabolic rates (kg./cal./hr.)

Subject	Rest (*a*)	Pedals driven by motor (*b*)	Rest (*c*)	Metabolic cost $(d) = b - \frac{1}{2}(a+c)$
1	83·9–108·2	104·4–166·3	76·6–91·6	13·2–74·2
		112·6–144·0		19·7–52·3
2	56·6– 71·1	68·2– 89·6	53·1–68·0	10·6–26·1
		88·6–128·0		30·3–64·9
3	70·5– 83·9	81·7–105·2	62·4–81·9	15·3–28·3
		97·7–110·9		24·5–41·6
4	62·7– 91·6	82·2–105·6	61·3–81·2	13·2–40·2
		90·7–112·6		18·9–31·3
5	72·7– 84·6	80·5– 98·9	62·5–71·8	7·0–24·4
		100·2–114·6		26·3–37·3

The upper figures in columns (*b*) and (*d*) give the range of values at 56 r.p.m., the lower figures at 74 r.p.m.

It will be noted that the metabolic rate at rest after the passive movement is less than that at rest before the movement. This was found quite consistently and is presumably due to the elapse of a further 1½ hr. since the previous meal. As the samples during passive movement are taken half-way through this period, the metabolic cost of the passive movement is computed by subtracting the mean of the values at rest before and after the movement from the metabolic rate during the passive movement.

The values given are, as far as it is possible to judge, close to the true values for passive leg rotation with the exception of that for subject 2 at 74 r.p.m. In

this case the subject found the movement very difficult indeed, and despite considerable effort was unable to relax to a degree subjectively comparable to that achieved at the slower rate.

DISCUSSION

The metabolic rate determined during passive leg rotation will vary inversely with the degree of relaxation achieved by the subject. Any resistance on the part of the subject to the pedal movement or any tendency to 'keep time' with the pedals will result in a greater muscle tone and a greater calorie expenditure. It follows, therefore, that, providing the subject co-operates effectively in maintaining relaxation of the leg muscles, the minimum value recorded will give the most satisfactory measure of the cost of truly passive movements.

Comparison with other values for passive cycling movements. It is difficult to compare the results reported here with those of Benedict & Cathcart, as they report only one experiment with a change from rest on the cycle to passive rotation of the legs on the same day, and the results of this experiment are unreliable because of belt slipping. They emphasize (1913, p. 118) that not only did their subject find the passive movements difficult, but even (pp. 117–18) that he became strained and uncomfortable while sitting on the cycle at rest. In the photograph given in Benedict & Cathcart's paper, the subject has his trunk inclined forwards, his arms bent at the elbow, and appears to be supporting the weight of his trunk mainly with his arms. My subjects were sitting upright and did not use their arms at all as a means of support. The position of the subject, which is determined by the structure of the bicycle, is probably an important factor in the comfort of the subject and the cost of passive movement.

The mechanical efficiency of cycling. The 'exercycle' used in this work could not be used as an ergometer, so that it was not possible to find the efficiency of the subjects. Garry & Wishart (1931) determined efficiency, using rest on the ergometer as the base-line, and found 20·0 and 21·1 % at 52 r.p.m. and 18·2 and 18·7 % at 70 r.p.m. for their two subjects. Adding the metabolic cost of passive movement reported here for subject 1 (who most closely resembles Garry & Wishart's subjects in size) to their base-line values, the efficiencies become 20·7, 21·7, 19·2 and 19·4 %. Similarly, Campbell *et al.* (1921) found a mean efficiency at 50 r.p.m., for two subjects at two loads, of 24·4 % using rest on the ergometer as base-line. This is increased to 25·2 % by adding the value reported here (subject 1) for passive movements.

The cause of the increased metabolism during passive movements. The metabolic cost of passive leg rotation is positive and increased by increasing the rate of leg movement. The cost of this passive movement may be due to (*a*) local factors in the legs, (*b*) changes in the rest of the body.

The local factors are (1) friction in the joints, (2) viscosity of tissues, including muscle, and (3) variation in muscle tone. In passive movement the force needed to overcome friction and viscosity is provided by the motor and would not appear as extra metabolism. It is probable, however, that some of this energy will be degraded to heat and will stimulate metabolism due to a local rise in temperature. Although this factor seems unlikely to be important in the present experiments, it would increase in importance at high rates of leg movement.

Some degree of tone exists in the muscles of any conscious subject, and stretching of these muscles during passive movements may result in increased metabolism.

Changes in the rest of the body caused by the leg movement may be due to the extra effort involved in maintaining the erect sitting posture, or to increased circulatory-respiratory movements resulting from the movement or from increased metabolism produced by the movement. The extra effort required to maintain the sitting position during passive leg movement can only be assessed subjectively and was not noticed by any of the subjects except subject 2 at the higher rate. In this case the difficulty was probably due to the fact that subject 2 was much shorter than the others, and that her legs were fully extended at the lowest position of the pedals. With this exception, the maintenance of posture did not seem to be an important factor in the metabolic cost at the moderate rates of leg rotation used.

Passive leg movements may increase respiration reflexly (Comroe, 1944) or by increased CO_2 production. The circulatory system may also be affected. In these experiments no records of circulatory changes were made, but the ventilation increased in all the experiments reported in Table 2 except with subject 4 at the slow rate, when it decreased. In this instance the extra metabolism must have been due to other causes, but in the other experiments the extra muscular effort involved in the increased ventilation certainly contributed to the increased metabolism.

SUMMARY

1. The metabolic rate of five subjects was determined before, during and after passive cycling movements, using motor-driven pedals on a special type of cycle. The metabolic cost of the movement was found by subtracting the mean of the values before and after from the value during the leg rotation.

2. The minimum metabolic cost in five subjects of passive cycling movements at 56 r.p.m. is between 7 and 15 kg.cal./hr./subject and at 74 r.p.m. between 19 and 26 kg.cal./hr./subject.

3. Using these figures as a base-line increases the value for mechanical efficiency in cycling by less than 1% compared with a base-line of rest on the cycle.

4. The increase in metabolism is probably due to increased tone in muscles and possibly to increased respiration and the extra effort required to maintain posture.

The author is grateful to Prof. D. Burns for his interest in the work, to the subjects for their patient co-operation, and especially to Mr A. Bone, whose technical skill and care contributed so much to the success of the experiments. A grant from the Research Fund of King's College is acknowledged with thanks.

REFERENCES

Benedict, F. G. & Cathcart, E. P. (1913). *Publ. Carneg. Instn*, no. 187.
Campbell, J. M. H., Douglas, C. G. & Hobson, F. G. (1921). *Philos. Trans.* B, **210**, 1.
Carpenter, T. M. (1939). *Publ. Carneg. Instn*, no. 303 B, 3rd ed.
Cathcart, E. P. & Cuthbertson, D. P. (1931). *J. Physiol.* **72**, 349.
Comroe, J. H., Jr. (1944). *Physiol. Rev.* **24**, 324.
Garry, R. C. & Wishart, G. M. (1931). *J. Physiol.* **72**, 425.
Hill, A. V. (1922). *J. Physiol.* **56**, 18.
Lindhard, J. (1915). *Pflüg Arch. ges. Physiol.* **161**, 233.

J. Physiol. (1949) 108, 359–361 612.824.492

OBSERVATION OF THE HYPOPHYSIO-PORTAL VESSELS OF THE LIVING RAT

By J. D. GREEN and G. W. HARRIS

From the Physiological Laboratory, University of Cambridge

(*Received* 13 *July* 1948)

The hypophysio-portal circulation was first described by Popa & Fielding (1930). They believed the blood in it flowed from the hypophysis to the hypothalamus. Wislocki & King (1936) and Wislocki (1938) suggested, on indirect evidence, that the blood flow is from the median eminence of the tuber cinereum to the pars distalis. Since Wislocki & King's work there has been much uncertainty as to the direction of flow in this system of vessels. Available evidence regarding the blood flow may be summarized: (1) histological appearance of the vessels (Wislocki & King, 1936; Green, 1948*a*); (2) observations on glands in which the vessels had been incompletely filled with india ink (Green & Harris, 1947); (3) the site of arrest of fat emboli within the system (Morato, 1939); (4) slow injection of india ink in dead animals with observation of filling of the vessels (Green, 1948*a*); (5) india ink injections made into the aorta of anaesthetized rats with intact, hemisected or transected pituitary stalks, followed by immediate decapitation. Observations on the filling of the vessels with ink indicated the blood flow to be towards the pars distalis (Harris, 1948); (6) direct observation of the direction of blood flow in the living animal.

Clearly the last type of evidence is the most satisfactory, but so far such observations have been confined to Amphibia. Houssay, Biasotti & Sammartino (1935) observed the direction of flow in *Bufo arenarum*, and Green (1947) has likewise seen the flow in the hypophysial vessels of anaesthetized amphibians (*Rana catesbiana*, *Ambystoma tigrinum*, *Triturus torosus* and *Necturus*). Recently (Green, 1948*b*) it has been found possible to expose the hypophysis in *Rana catesbiana* under procaine anaesthesia and observe the course of blood flow for many hours. Since the operation is nearly bloodless and the animals are conscious, it is felt that conditions are almost ideal. As far as is known, no observations have been made on the circulation in living mammals.

METHODS

Rats were used since they possess long hypophysio-portal vessels, in a horizontal plane, which are readily approached and easily observed. Adult animals were anaesthetized with ether and tracheotomized. The facial artery and vein were ligated near the angle of the jaw and clamped dorsal to the angle of the mouth. The lower jaw was divided on either side through the angle and removed. As soon as haemostasis had been secured the soft palate was incised and removed except for a narrow rim. The periosteum of the skull was stripped beneath the sphenoid and anterior part of the basiocciput. The bone was then picked away with a dental scaling pick. A transverse venous sinus lies in the sphenoid beneath the stalk. This was opened and packed with beeswax. As soon as a clean field had been obtained the cancellous bone was scraped away until the shiny inner table could be seen. The remaining bone was then carefully picked away under a binocular dissecting microscope to expose the dura. The dura was pricked and the hole extended by tearing. For observation of the vessels an ordinary microscope was used ($\frac{2}{3}$ objective and $\times 9$ eyepiece, with oblique illumination from above).

RESULTS

A satisfactory exposure of the portal vessels was secured in twelve rats. It was seen that the median eminence of the tuber cinereum and pituitary stalk are clearly demarcated from the surrounding hypothalamus by their extreme vascularity. The rich capillary network of the median eminence extends into the pituitary stalk and collects to form the large portal trunks which may be observed to fan out into the sinusoids of the pars distalis. In all twelve rats the blood flow was seen to be from the median eminence towards the pars distalis. Under the $\frac{2}{3}$ objective the red blood corpuscles within the vessels were readily observed. In many cases the animals were in excellent condition and only lightly anaesthetized. A few, however, showed signs of surgical shock and an occasional vessel appeared thrombosed. These signs of damage were not associated with any change in direction of flow. The blood stream in the vessels appeared comparable in rate to that in the hypothalamic veins. The flow shows no signs of pulsation and is always of a uniform character. All the large vessels of the hypophysial stalk appear to carry blood caudally to the pars distalis from the dense capillary plexus of the median eminence. They cannot in any sense be regarded as T-shaped branches from the carotid artery.

DISCUSSION

These observations accord well with the concept of neurovascular control of the pars distalis (Harris, 1944; Green & Harris, 1947). Histological evidence indicates that a hypophysio-portal circulation is a constant feature in vertebrates from the Salientia to the Primates (Green, 1948*c*), even in those species in which the pars distalis is separated from the neurohypophysis by a dural septum (Harris, 1947; Green, 1948*b*).

It is felt that the above data provide the most direct and satisfactory evidence that the vessels of the hypophysial stalk are true portal vessels carrying blood from the median eminence of the tuber cinereum to the pars distalis.

SUMMARY

1. A method of exposing the hypophysio-portal blood vessels of the living rat is described.

2. The direction of the blood flow in these vessels is from the median eminence of the tuber cinereum to the pars distalis.

REFERENCES

Green, J. D. (1947). *Anat. Rec.* **99**, 21.
Green, J. D. (1948*a*). *Anat. Rec.* **100**, 273.
Green, J. D. (1948*b*). Unpublished observation.
Green, J. D. (1948*c*). Proc. Anat. Soc., *J. Anat., Lond.* (in the Press).
Green, J. D. & Harris, G. W. (1947). *J. Endocrinol.* **5**, 136.
Harris, G. W. (1944). Thesis for M.D. degree, Cambridge University.
Harris, G. W. (1947). *Nature, Lond.*, **159**, 874.
Harris, G. W. (1948). Unpublished observation.
Houssay, B. A., Biasotti, A. & Sammartino, R. (1935). *C.R. Soc. Biol., Paris*, **120**, 725.
Morato, M. J. X. (1939). *Anat. Rec.* **74**, 297.
Popa, G. T. & Fielding, U. (1930). *J. Anat., Lond.*, **65**, 88.
Wislocki, G. B. (1938). *Res. Publ. Ass. nerv. ment. Dis.* **17**, 48.
Wislocki, G. B. & King, L. S. (1936). *Amer. J. Anat.* **58**, 421.

J. Physiol. (1949) 108, 362–364 612.174

THE CAUSES OF SERUM BRADYCARDIA

By G. S. DAWES and W. FELDBERG

From the Department of Pharmacology and The Nuffield Institute for Medical Research, University of Oxford

(*Received* 15 *July* 1948)

Brodie (1900) showed that in cats an intravenous injection of serum produces bradycardia which is abolished by cutting the vagi and which he attributed solely to a reflex, the receptors for which were located in the pulmonary circulation. Recently reflex bradycardia has been obtained not only from the lungs but also from receptor areas in the vicinity of the ventricles by the veratrum alkaloids and nicotine (Dawes, 1947) and by adenosine triphosphate (Emmelin & Feldberg, 1948). We have been able to show that this mechanism is also partly responsible for the reflex bradycardia produced by the injections of horse serum into cats.

RESULTS

In all experiments an intravenous injection of 0·2–1 ml. of horse serum into cats under chloralose anaesthesia produced a fall in arterial blood pressure with pronounced slowing of the heart. The effect was obtained repeatedly in the same animal if an interval of 15 min. or longer was left between each injection. The bradycardia (which was abolished by cutting the vagi) was due to reflexes originating partly from the lungs, as stated by Brodie, and partly from the heart. When injections of the same amounts of serum were made into the right and left atria alternatively, slowing in heart rate occurred about 1 sec. earlier on injection into the left atrium than on injection into the right atrium, but the effect on injection into the latter was stronger and obtained with quantities of serum too small to cause bradycardia on injection into the left atrium. This shows that part at least of the reflex bradycardia must originate in that part of the circulatory system which lies between the right and left atria, i.e. the lungs, but that a further component of the reflex originates in that part of the circulatory system which lies distal to the left atrium. The mechanism of this latter bradycardia resembles that of the veratrum alkaloids, and has its receptors in the vascular field supplied by the coronary arteries. Whereas injections of horse serum into the left ventricle of a cat with open thorax caused the characteristic fall of blood pressure and heart rate, injection of the

same dose into the ascending aorta above the origin of the coronary arteries had no effect of this kind. Direct evidence for the origin of part of the reflex bradycardia from the heart was obtained when injections of small doses of horse serum were made into the left coronary artery which was cannulated and perfused according to the technique described by Dawes (1947).

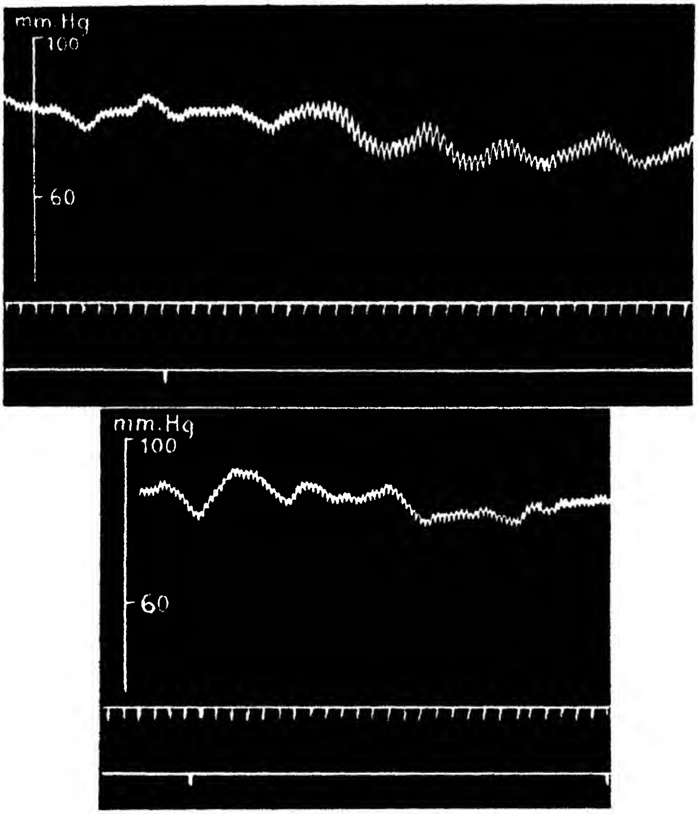


Fig. 1. Femoral blood pressure; a cat under chloralose anaesthesia. Time, 1 sec. Above, injection of 0·05 ml. horse serum into perfused left coronary artery causes slowing of the heart from 240 to 180 beats per minute. Below, injection of same quantity into cavity of right ventricle does not affect the heart rate.

In the figure the injection of 0·05 c.c. of horse serum into the left coronary artery produced a slowing of the heart rate from 240 to 180 beats per minute and a slight fall of arterial blood pressure, whereas the same injection into the right ventricle produced no visible cardio-vascular effects. In this cat the receptors in the heart appeared, therefore, to be more sensitive to the horse serum than those in the lungs; this was not the case in all animals.

SUMMARY

The strong bradycardia produced by injections of serum into cats ('Brodie effect') has been attributed to a reflex from the lungs.

Experiments with horse serum have shown that there are in addition receptor areas in the vicinity of the heart ventricles, stimulation of which contributes to the bradycardia.

This work was done while one of us (G. S. D.) was Foulerton Research Fellow of the Royal Society, and the other (W. F.) was in receipt of a grant from the Medical Research Council.

REFERENCES

Brodie, T. G. (1900). *J. Physiol.* **26**, 48.
Dawes, G. S. (1947). *J. Pharmacol.* **89**, 325.
Emmelin, N. & Feldberg, W. (1948). *Brit. J. Pharmacol.* **3**, 273.

J. Physiol. (1949) 108, 365–373 612.82.026:546.46

THE INFLUENCE OF MAGNESIUM ON RESPIRATION, GLYCOLYSIS AND CHOLINESTERASE ACTIVITY IN RAT BRAIN*

By C. N. PEISS, V. E. HALL AND J. FIELD*

From the Department of Physiology, Stanford University School of Medicine

(*Received* 19 *July* 1948)

The thermoregulatory behaviour of the higher vertebrates is capable of achieving a fair balance between heat production and heat loss over a rather wide range of environmental temperature. The chief locus of this behaviour is the temperature regulating centre of the hypothalamus (Ranson, 1940). There appears to be a normal set level or range for this centre because under usual conditions the body temperature of homoiotherms lies between 37 and 40° C. (Evans & Starling, 1945). Some temperature-sensitive process or processes within the neurones of the centre must be so organized as to yield this set level. The nature of this process is not known. If it be assumed that some feature of the local cellular metabolism of the centre is a factor determining the level, some information concerning the regulatory process might be obtained by the use of agents which influence both body temperature and cell metabolism.

Magnesium appears to be such an agent. The antipyretic action of magnesium salts has been known for many years (Schütz, 1916; Barbour & Winter, 1928; Winter & Barbour, 1928; Taylor & Winter, 1929; Steadman, Ariel & Warren, 1943; Sunderman & Haymaker, 1947; Sollman, 1948). High serum-magnesium levels are found in hibernating animals (Lustig, Ernst & Reuss, 1937; Suomaleinen, 1938, 1939), in which body temperature is reduced and thermoregulation is impaired but not abolished (Benedict & Lee, 1938; Suomaleinen, 1939). Recently Heagy & Burton (1947, 1948) reported that administration to dogs of subnarcotic doses of magnesium chloride caused lowering of the rectal temperature whether the animal was in an environment that was warm, cool or at normal room temperature. These observations, together with unpublished findings in this laboratory, are in harmony with the working hypothesis that the antipyretic action of magnesium involves a lowering of the set level of the thermoregulatory centre.

* This investigation was carried out under a Contract between the Air Materiel Command, Wright Field, Dayton, Ohio, and Stanford University.

Magnesium is known to play a part in several intracellular processes. For example, it is an important factor in a number of the sequential enzymatic reactions of glycolysis (Cori, 1942; Sumner & Somers, 1943; Barron, 1943) which are common to many types of cells including those of mammalian brain (Banga, Ochoa & Peters, 1939; Ochoa, 1940, 1941; Macfarlane & Weil-Malherbe, 1941; Gerard, 1946). Moreover, magnesium is known to activate cholinesterase (Bodansky, 1946) and to enhance the ability of the metabolic processes to increase the *L* fraction of the membrane potential of the nerve fibre (Lorente de Nó, 1947). In view of the nature of the antipyretic action of magnesium (Heagy & Burton, 1948) and of the significance of this substance in cell metabolism, it appeared desirable to examine the effects of graded concentrations of magnesium on the metabolism of the thermoregulatory centre. Technical difficulties precluded this direct approach. However, because of the qualitative similarity of the metabolic pattern in the several parts of the central nervous system (Page, 1937), quantitative differences appearing in one region might well be paralleled by changes in a similar direction in other regions. The positive correlation between fall in rectal temperature and apparent general depression of the central nervous system reported by Heagy & Burton (1948) may be cited as evidence favouring this view. Accordingly, the influence of magnesium on respiration and anaerobic glycolysis in rat cerebral cortex slices and on cholinesterase activity in whole rat brain homogenates has been investigated.

METHODS

Adult albino rats of the Slonaker-Wistar strain were used. The brain was rapidly removed after decapitation and cerebral cortex slices were prepared by the cold moist box technique which has been described previously (Field, 1948; Peiss & Field, 1948). By this procedure the tissue is kept in a cold moist environment from the time of excision until the respirometer flasks, suitably loaded, are placed in a constant temperature bath. Thus in studies on oxygen consumption imbalance between the anaerobic and aerobic phases of metabolism is minimized during the period of tissue manipulation (Fuhrman & Field, 1943, 1945) and in preparation for anaerobic work the overall metabolism is kept at a low level until suitable conditions are provided for the supply of nutrients and the removal of metabolic end-products. Control experiments showed that under the conditions of these experiments the oxygen consumed or the carbon dioxide produced was proportional to the *initial* wet weight of tissue over the range 10–90 mg. Most samples weighed from 40 to 50 mg. Slice thickness was 0·4–0·5 mm. (Field, 1948). Aliquot samples were placed in small weighing bottles and dried to constant weight in an electric oven at 105° C. Manometric measurements were made in a constant temperature bath at 37·5±0·01° C. after 15 min. of thermoequilibration.

Respiration was measured by the Warburg manometric method (Dixon, 1943; Umbreit, Burris & Stauffer, 1945, Field, 1948). The respirometers were flushed with oxygen before being placed in the constant temperature bath. Thus the gas phase was oxygen. The centre wells contained 5% KOH and were fitted with Whatman No. 40 filter paper wicks. The liquid phase was Ringer-phosphate solution containing 0·011 M-glucose (Krebs, 1933). The sidearms contained graded amounts of magnesium chloride made up in the same solution. The contents of the sidearms were added to the main compartments of the flasks after a 30 min. control period, so that control and 'magnesium added' runs were made with the contents of each flask.

Anaerobic glycolysis was measured by the manometric method (Dixon, 1943; Umbreit *et al.* 1945). The gas phase was 95% N_2–5% CO_2 which had been passed through a hot copper tube to remove traces of oxygen (Savage & Ordal, 1940). Uniform gassing was effected by passing this mixture through the respirometers in series. After leaving the last respirometer the gas was passed through a Wolff bottle so that the rate of flow could be observed. Care was taken to make sure that a good steady flow of gas was maintained for 10 min. after the respirometers had been placed in the constant temperature bath. The liquid phase was Ringer-bicarbonate solution (Krebs & Henseleit, 1932). This medium had previously been gassed for over an hour with the same oxygen-free mixture used as the gas phase in the respirometers. The medium contained glucose in a final concentration of 0·011 M and magnesium chloride in concentrations ranging up to $43{\cdot}7 \times 10^{-3}$ M. Thus there was no 'pre-addition' control period as in the case of oxygen-consumption measurements. This procedure was followed to avoid the disturbance in the rate of CO_2 evolution produced by the handling necessary to make additions from the sidearms of the vessel.

Cholinesterase activity was measured manometrically (Nachmansohn & Feld, 1947; DuBois & Mangun, 1947). The enzyme extract used was the 'Supernatant fraction' of Nachmansohn & Feld (1947). This was prepared from whole rat brain homogenized in ice-cold calcium-free Ringer-bicarbonate solution in a Waring blendor. The main compartments of the respirometer flasks contained the enzyme preparation in the calcium-free Ringer-bicarbonate solution together with the desired amounts of magnesium chloride. The sidearms contained acetylcholinechloride (Merck) made up in the same solution. The contents of the sidearms were added to the main compartments at the end of thermoequilibration. The final concentration of acetylcholine was 0·015 M. The gassing procedure was the same as in the experiments on anaerobic glycolysis.

Respiration and glycolysis data are expressed in the conventional 'Q' notation. Thus Q_{O_2} and $Q_A^{N_2}$ denote respectively microlitres of oxygen consumed and microlitres of acid produced, measured as gas under standard conditions per mg. *initial* dry weight of tissue per hour (cf. Burk, Sprince, Spangler, Kabat & Furth, 1941). The superscript 'N_2' denotes the gas mixture 95% N_2 + 5% CO_2. In all cases Q_{O_2} was constant for several hours and $Q_A^{N_2}$ for 40–60 min. The values of oxygen consumption and glycolysis presented in the figures and tables were calculated from measurements made during steady state periods. Cholinesterase activity is expressed in terms of mg. of acetylcholine hydrolysed in 1 hr. by 1·0 ml. of supernatant fraction from a homogenate containing 100 mg. of fresh tissue per ml. Readings were taken at 5 min. intervals for a period of 40 min.

RESULTS

Respiration. It is shown in Fig. 1 that the respiration of rat cerebral cortex slices was not affected by magnesium-ion concentration up to $8{\cdot}75 \times 10^{-3}$ M. As the magnesium-ion concentration was increased above this level there was a moderate inhibition of respiration, amounting to about 20% at the highest concentration used ($29{\cdot}2 \times 10^{-3}$ M). Thus, under the conditions of these experiments, the concentration of magnesium ion in the liquid phase of the respirometer flasks was not a factor modifying the oxygen consumption of cerebral cortex slices until a concentration well above the physiological range in serum was used (cf. Suomaleinen, 1939).

Anaerobic glycolysis. Fig. 2 illustrates the effects of graded concentrations of magnesium ion on anaerobic glycolysis in rat cerebral cortex slices. It is evident from inspection of Fig. 2 that anaerobic glycolysis is decidedly influenced by the level of magnesium-ion concentration in the suspension medium. As this level was varied over the range 0 to $12{\cdot}5 \times 10^{-3}$ M there was a progressive rise

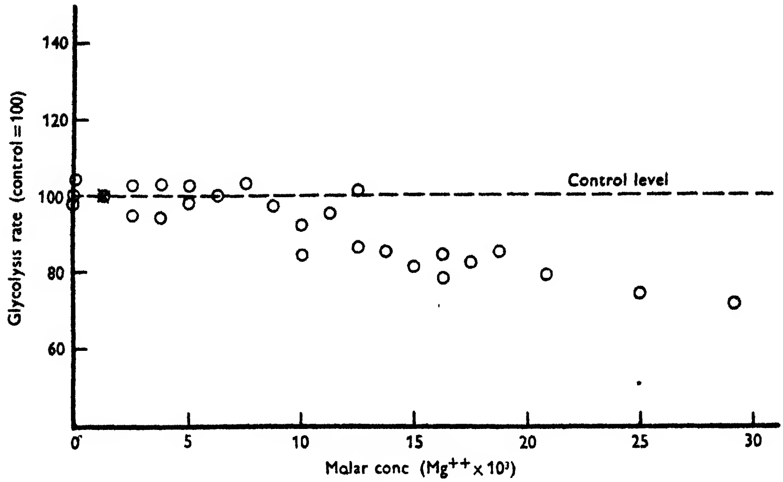


Fig. 1. Effect of graded concentrations of magnesium on the oxygen consumption of rat cerebral cortex slices. Suitable amounts of magnesium chloride, made up in 0·2 ml. Krebs Ringer solution, were added from the sidearms of the respirometer flasks after a 30 min. control period. Main compartments of the flasks contained 1·8 ml. of Krebs Ringer solution without magnesium. Oxygen consumption was a rectilinear function of time both before and after additon of magnesium. Further details are given in Tables 1 and 2.

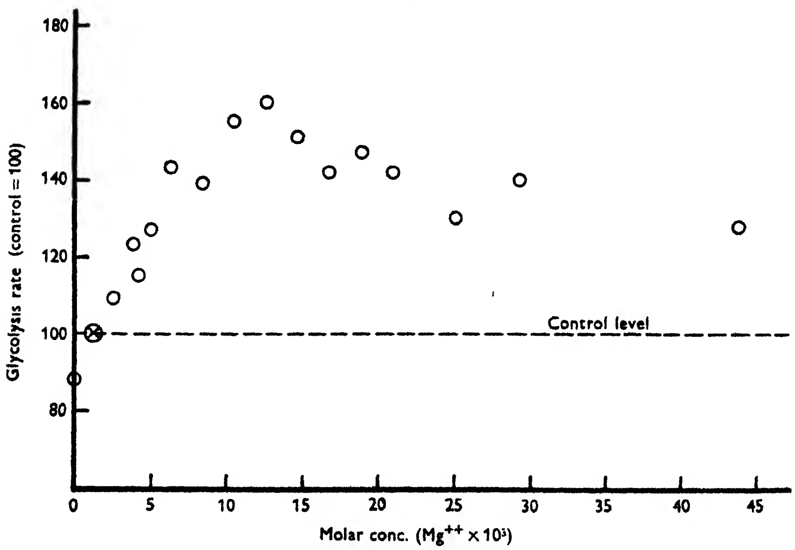


Fig. 2. Effect of graded concentrations of magnesium on anaerobic glycolysis in rat cerebral cortex slices. Main compartment of the respirometer flasks contained 2·0 ml. of Krebs Ringer-bicarbonate solution, with varied concentrations of magnesium chloride. No additions were made from sidearms since such additions disturb the rate of glycolysis for a time. Rate of glycolysis was fairly constant for the first 40 min. All calculations are based on readings taken during this 'steady state' period. Further details are given in Table 2.

in the rate of glycolysis to a maximum 60% above the rate of the control (containing $1 \cdot 19 \times 10^{-3}$ M-magnesium). Further increase in magnesium concentration led to a decrease in glycolysis from this maximum, but at the highest concentration tested ($43 \cdot 7 \times 10^{-3}$ M) the glycolytic rate was still approximately 30% above the control level.

When the data on which Fig. 2 is based were treated statistically it was found that the variations in $Q_A^{N_2}$ due to concentration of magnesium are highly significant (Snedecor, 1946; analysis of variants). Similar treatment revealed that the interaction of differences in readings with time were not significant, i.e. that steady states were obtained.

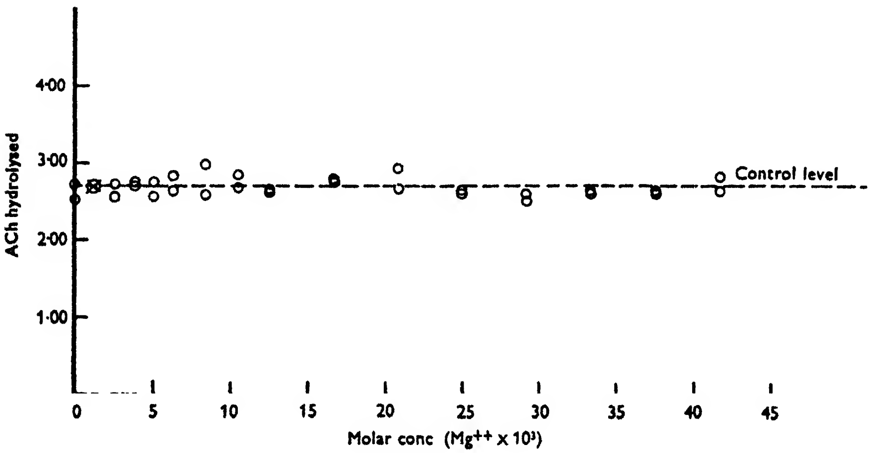


Fig. 3. Effect of graded concentrations of magnesium on the hydrolysis of acetylcholine by the supernatant fraction of whole rat brain homogenates. Main compartment of the respirometer flasks contained 0·2 ml. of the supernatant fraction of the homogenate and 1·15 ml. of calcium-free Krebs Ringer-bicarbonate solution, made up to contain the desired amounts of magnesium chloride. Sidearms contained 0·15 ml. of acetylcholine chloride (Merck) in a final concentration of 0·015 M. After thermoequilibration the contents of the sidearms were added to the main compartments. Crossed circle represents the control level ($1 \cdot 19 \times 10^{-3}$ M-magnesium). Open circles denote the means of duplicate determinations at the indicated concentrations of magnesium.

Cholinesterase activity. Under the conditions of these experiments the cholinesterase activity of the supernatant fraction of whole rat brain homogenate (Nachmansohn & Feld, 1947) was not affected by the concentration of magnesium in the suspension medium over the range 0 to $41 \cdot 7 \times 10^{-3}$ M. This is shown in Fig. 3.

Collected data. The data used in the construction of Figs. 1–3, together with the absolute values of the means of the control observations and additional statistical information, are presented in Tables 1 and 2. By means of these figures it is possible to convert any of the percentage values of respiration,

glycolysis or cholinesterase activity to the corresponding absolute rates, thus facilitating comparison with other observations.

TABLE 1. Summary of data showing the effect of graded concentrations of magnesium on the rate of anaerobic glycolysis, the rate of oxygen consumption and the rate of acetylcholine hydrolysis in rat brain

(Control level is at a magnesium concentration of $1 \cdot 19 \times 10^{-3}$M. Figures in parentheses represent the number of determinations. All other values are means of duplicate determinations.)

Molar conc. ($Mg^{++} \times 10^{-3}$)	$Q_A^{N_2}$ as % of control	Q_{O_2} as % of control	ACh hydrolysis as % of control
0·00	88 (6)	100, 98, 104	97·5
1·19	100 (30)	100 (62)	100·0 (12)
2·50	109 (3)	94, 102	98·0
3·75	123 (3)	94, 103	100·5
4·17	115 (3)	—	—
5·00	127 (3)	98, 102	98·5
6·25	143 (6)	100	101·5
7·50	—	103	—
8·33	139 (6)	—	103·5
8·75	—	97	—
10·00	—	84, 92	—
10·42	155 (6)	—	102·0
11·25	—	95	—
12·50	160 (6)	86, 101	97·5
13·75	—	85	—
14·58	151 (3)	—	—
15·00	—	81	—
16·25	—	78, 84	—
16·67	142 (3)	—	103·0
17·50	—	82	—
18·75	147 (6)	85	—
20·83	142 (3)	79	104·0
25·00	130 (3)	74	97·0
29·17	140 (3)	71	94·5
33·33	—	—	97·5
37·50	—	—	97·5
41·67	—	—	101·5
43·75	128 (3)	—	—

TABLE 2. Means and other statistics of absolute values of control Q_{O_2}, $Q_A^{N_2}$ and cholinesterase activity (ACh activity)

(Units defined in section on methods.)

	Q_{O_2}	$Q_A^{N_2}$	ACh activity
No. of animals	12	12	6
No. of samples	62	30	12
Mean	10·94	7·53	2·69
Range	9·27–12·80	5·03–11·05	2·52–2·84
Standard deviation	0·830	1·731	0·0826

DISCUSSION

Concentration-action curves (Clark, 1933), illustrating the influence of graded concentrations of magnesium in the suspension medium on the oxygen consumption and anaerobic glycolysis of rat cerebral cortex slices and on the cholinesterase activity of the supernatant fraction of whole rat brain homogenate, have not been described previously, as far as the authors know. However, data of this sort are available for rat diaphragm respiration (Stadie &

Zapp, 1947), the respiration of minced pigeon-breast muscle (Elsden, 1939) and for glycolysis in rat brain extracts (Geiger, 1940).

The present results on the respiration of cerebral cortex slices are in agreement with those of Stadie & Zapp (1947) on diaphragm in that concentrations of magnesium above 10×10^{-3} M depress oxygen uptake. It is interesting to note that inhibition of the respiration of rat cerebral cortex slices begins at magnesium concentrations corresponding quite closely with serum-magnesium levels producing deep anaesthesia in the rabbit ($7{\cdot}38$ to $8{\cdot}41 \times 10^{-3}$ M; Taylor & Winter, 1929). However, serum-magnesium levels which impair temperature regulation in the dog (*c.* 10 mg. % or $4{\cdot}1 \times 10^{-3}$ M; Heagy & Burton, 1948) lie in the range of concentrations of magnesium in the suspension medium which do not influence rat cerebral cortex slice respiration (Fig. 1). Thus the present observations on the influence of graded concentrations of magnesium on the respiration of cerebral cortex slices do not provide a satisfactory clue as to a possible influence of subnarcotic doses of magnesium on the metabolism of the thermoregulatory centre. The same comment holds for the results of the measurements of cholinesterase activity as a function of the concentration of magnesium in the suspension medium (Fig. 3).

The most interesting lead is provided by the observations on anaerobic glycolysis as a function of the concentration of magnesium in the suspension medium. It is shown in Fig. 2 that magnesium concentrations of 3 to 7×10^{-3} M caused a striking increase in glycolysis in cerebral cortex slices. These concentrations correspond to the range of serum magnesium levels which cause a decrease in rectal temperature in the dog, possibly by lowering the set level of the thermoregulatory centre (Heagy & Burton, 1948; Hall *et al.* unpublished observations). Thus if there is a key regulatory process in the cells of the centre which is affected by the concentration of magnesium, this process may be one of the reactions of the anaerobic phase of carbohydrate breakdown. However, no proof is available that this is so. The evidence is suggestive only, and it is quite possible that the increase in anaerobic glycolysis of cerebral cortex slices with increase in the concentration of magnesium ion in the suspension medium is in no way related to the effects of magnesium on temperature regulation.

SUMMARY

1. The oxygen consumption and anaerobic glycolysis of rat cerebral cortex slices and the cholinesterase activity of the supernatant fraction of whole rat brain have been measured at different levels of magnesium concentration in the suspension medium.

2. Magnesium concentrations up to $8{\cdot}75 \times 10^{-3}$ M did not affect the rate of oxygen consumption of cerebral cortex slices suspended in Krebs Ringer-phosphate solution. Higher concentrations, up to $29{\cdot}2 \times 10^{-3}$ M, caused moderate lowering of the rate of oxygen uptake.

3. There was an increase in the rate of anaerobic glycolysis in rat cerebral cortex slices suspended in Krebs-Henseleit Ringer-bicarbonate solution as the magnesium concentration in the medium was raised from 0 to $12\cdot5 \times 10^{-3}$ M. The maximum rate, attained when the magnesium concentration was $12\cdot5 \times 10^{-3}$ M, was 60% above the rate at the control level of magnesium ($1\cdot19 \times 10^{-3}$ M). Higher concentrations lowered the rate of glycolysis from the peak value, but at $43\cdot7 \times 10^{-3}$ M glycolysis was still about 30% above the control rate.

4. Concentrations of magnesium over the range of 0 to $41\cdot7 \times 10^{-3}$ M did not influence the cholinesterase activity of the supernatant fraction of whole rat brain homogenate made up in calcium-free Ringer-bicarbonate solution.

5. It is suggested that the influence of magnesium on anaerobic glycolysis in central nervous system tissue may provide a clue to the mechanism of the influence of magnesium on temperature regulation in homoiotherms.

REFERENCES

Banga, I., Ochoa, S. & Peters, R. A. (1939). *Biochem. J.* **33**, 1980.

Barbour, H. G. & Winter, J. E. (1928). *Proc. Soc. exp. Biol., N.Y.*, **25**, 582.

Barron, E. S. G. (1943). *Advances in Enzymol.* **3**, 149.

Benedict, F. G. & Lee, R. C. (1938). *Publ. Carneg. Instn*, no. 497.

Bodansky, O. (1946). *Ann N.Y. Acad. Sci.* **47**, 521.

Burk, D., Sprince, H., Spangler, J. M., Kabat, E. A. & Furth, J. (1941). *J. Nat. Cancer Inst.* **2**, 201.

Clark, A. J. (1933). *The Mode of Action of Drugs on Cells.* Baltimore: Williams and Wilkins.

Cori, C. F. (1942). *A Symposium on Respiratory Enzymes*, p. 175. Madison: Univ. of Wisconsin Press.

Dixon, M. (1943). *Manometric Methods.* London: Cambridge Univ. Press.

DuBois, K. P. & Mangun, G. H. (1947). *Proc. Soc. exp. Biol., N.Y.*, **64**, 137.

Elsden, S. (1939). *Biochem. J.* **33**, 1890.

Evans, C. L. & Starling, E. H. (1945). *Human Physiology*, p. 997. Philadelphia: Lea and Febiger.

Field, J. (1948). *Methods in Medical Research*, **1**. Chicago: Year Book Publ. 1948.

Fuhrman, F. A. & Field, J. (1943). *Amer. J. Physiol.* **139**, 193.

Fuhrman, F. A. & Field, J. (1945). *J. biol. Chem.* **153**, 515.

Geiger, A. (1940). *Biochem. J.* **34**, 465.

Gerard, R. W. (1946). *Ann. N.Y. Acad. Sci.* **47**, 575.

Heagy, F. C. & Burton, A. C. (1947). *Federation Proc.* **6**, 126.

Heagy, F. C. & Burton, A. C. (1948). *Amer. J. Physiol.* **152**, 407.

Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **217**, 191.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

Lorente de Nó, R. (1947). *A Study of Nerve Physiology. Stud. Rockefeller Inst. med. Res.* **132**, 133.

Lustig, B., Ernst, T. & Reuss, E. (1937). *Biochem. Z.* **290**, 95.

Macfarlane, M. G. & Weil-Malherbe, H. (1941). *Biochem. J.* **35**, 1.

Nachmansohn, D. & Feld, E. A. (1947). *J. biol. Chem.* **171**, 715.

Ochoa, S. (1940). *Nature, Lond.*, **145**, 747.

Ochoa, S. (1941). *J. biol. Chem.* **141**, 245.

Page, I. H. (1937). *Chemistry of the Brain.* Springfield: C. C. Thomas.

Peiss, C. N. & Field, J. (1948). *J. biol. Chem.* **175**, 49.

Ranson, S. W. (1940). *Res. Publ. Ass. nerv. ment. Dis.* **20**, 342.

Savage, G. M. & Ordal, Z. J. (1940). *Science*, **91**, 222.

Schütz, J. (1916). *Arch. exp. Path. Pharmak.* **79**, 285.

Snedecor, G. W. (1946). *Statistical Methods.* Ames: Iowa State College Press.

Sollman, T. (1948). *A Manual of Pharmacology.* Philadelphia: W. B. Saunders.

Stadie, W. C. & Zapp, J. A., Jr. (1947). *J. biol. Chem.* **170**, 55.

Steadman, L. T., Ariel, I. & Warren, S. L. (1943). *Cancer Res.* **3**, 471.

Sumner, J. B. & Somers, G. F. (1943). *Chemistry and Methods of Enzymes.* New York: Academic Press.

Sunderman, F. W. & Haymaker, W. (1947). *Amer. J. med. Sci.* **213**, 562.

Suomaleinen, P. (1938). *Nature, Lond.*, **141**, 471.

Suomaleinen, P. (1939). *Ann. Acad. Scient. Fennicae*, **53**, no. 7, p. 1.

Taylor, W. F. & Winter, J. E. (1929). *J. Pharmacol.* **35**, 435.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis: Burgess Co.

Winter, J. E. & Barbour, H. G. (1928). *Proc. Soc. exp. Biol., N.Y.*, **25**, 587.

J. Physiol. (1949) 108, 375–397 546.185:612.817.612.833

ACTION OF CONDENSED ALKYL PHOSPHATES ON THE NERVE-MUSCLE PREPARATION AND THE CENTRAL NERVOUS SYSTEM OF THE CAT

By MARY CHENNELLS, W. F. FLOYD AND SAMSON WRIGHT

From the Department of Physiology, Middlesex Hospital Medical School, London

(*Received* 11 *March* 1948)

A preliminary report on the general pharmacological actions of two condensed alkyl phosphates, hexaethyltetraphosphate ('HETP') and tetraethylpyrophosphate ('TEPP') has already been published (Burgen, Keele, Chennells, del Castillo, Floyd, Slome & Wright, 1947); details about the chemical properties of the drugs were communicated by Topley (1947). In this paper we shall describe the action of these drugs on the nerve-muscle response and on certain reflexes in the cat. The actions of the drugs on other systems are fully described by Burgen, Keele & Slome (1949).

The exact constitution of HETP is at present uncertain. It is known to be a mixture of ester phosphates, polyphosphates and perhaps metaphosphates. On solution in water an immediate hydrolysis occurs and it is possible that triethylpyrophosphate is formed and is the physiologically active molecule. A further slow hydrolysis occurs forming equimolecular proportions of monoethyl phosphoric and diethyl phosphoric acids, which are physiologically inert. Other workers (Hall & Jacobson, 1948) have suggested that the biological activity of HETP is due to a content of 20% TEPP.

METHODS

Cats were used under chloralose anaesthesia (0·08 g./kg. body weight) or after decerebration at the mid-collicular level; in the latter case at least 1 hr. was allowed to elapse to enable the effects of the initial chloroform—ether anaesthesia to wear off. In some experiments the chloralosed or decerebrate cat was subsequently made spinal by a trans-section in the mid-thoracic region. Nerve-muscle and reflex responses were elicited and recorded as described below after appropriate fixation of the limb by means of drills. For nerve stimulation Collison electrodes were sometimes used, but in most experiments open electrodes of silver wire mounted in perspex were employed.

Nerve muscle. The femoral nerve-quadriceps preparation was employed using both mechanical (torsion lever) and electrical recording. Muscle-action potentials were picked up by belly-tendon leads and amplified by a resistance-capacity coupled push-pull amplifier. The action potentials were displayed on a 40 msec. sweep on the cathode ray oscilloscope and photographed so that each sweep appears as an oblique line across the photographic record, with the action potential deflexions parallel to the direction of movement of the paper. An electronic stimulator was used with an air cored coil, giving supra-maximal stimuli which were applied to the motor nerve at rates between 1 in 10 sec. and 100 pulses per sec.

Flexor reflex. Mechanical recording (torsion lever) from the anterior tibial muscle of one side was employed. The central end of the cut ipsilateral popliteal nerve was stimulated with a second electronic stimulator of the type referred to above.

Knee jerk. This was recorded mechanically with a torsion lever in response to the stimulus applied by an automatic knee jerk hammer (Schweitzer & Wright, 1937*a*) which struck the patellar tendon at regular intervals (once in 10 sec.).

Crossed extensor reflex. Movements of the distal part of the leg employed for the knee jerk were recorded in response to stimulation of the central end of the contralateral popliteal nerve as described for the flexor reflex (see above).

Other responses were studied occasionally, e.g. ipsilateral reflex response of m. peroneus longus, or reflex response of various muscles to table-banging.

The drugs used were injected intravenously (into the central end of the jugular vein), intra-arterially (into the central end of the inferior mesenteric or iliac arteries (Wilson & Wright, 1936–7), or intrathecally (Calma & Wright, 1947) approximately at the level of the seventh lumbar vertebra. The HETP and TEPP were diluted with saline just before being injected; in some experiments the dilutions were made from the original liquid, and in others from a 1% stock solution of the drug in propylene glycol.

RESULTS

In the cat, HETP and TEPP have, generally speaking, qualitatively similar effects as judged by the responses of the nerve muscle preparation and the reflexes; TEPP is, however, about five to ten times as powerful as HETP. The effects make their appearance after a latency which varies inversely with the dose employed. With large doses the maximal effects appear within a few seconds to a few minutes; with threshold doses the delay may be up to 5–10 min. The drugs initially potentiate the nerve-muscle response and enhance the reflexes; with larger doses depression usually develops. The effect on the reflexes is in part due to a direct action on the central nervous system (spinal cord), for intrathecal injections modify the reflexes at a time when the drug has produced no changes in peripheral structures.

Action on the nerve-muscle preparation

Response to single supramaximal motor-nerve stimuli. Typical mechanical responses of the femoral nerve-quadriceps preparation in the cat under chloralose anaesthesia are shown in Figs. 1 and 2. Records on the slow drum illustrate changes in contraction tension. Records on the fast drum show the changes in the shape of the contraction curve. In Fig. 1, ½–1 min. after an intravenous injection of 1 mg. of HETP, there was an increase in the tension of the muscle response which rose to a maximum of 40% above the control level within 2 min. The tension was still 20% above the control level after 24 min. The fast records show a considerable prolongation of the response. Very slight fibrillar twitching developed. A second intravenous injection of 1 mg. of HETP produced a trivial further increase in contraction tension and an increase in the size and extent of the fibrillar twitching which was soon followed by a marked depression in the height of the nerve-muscle response. Some recovery took place after 15 min. but the tension was then still below the control level.

A further dose of 5 mg. of HETP injected 70 min. after the first and 46 min. after the second injection produced a profound and lasting depression of the response accompanied by an intensification of the twitching.

Fig. 2 illustrates similar changes produced by TEPP. In this experiment, at the peak of the response, the duration of the muscle curve was 2 sec.

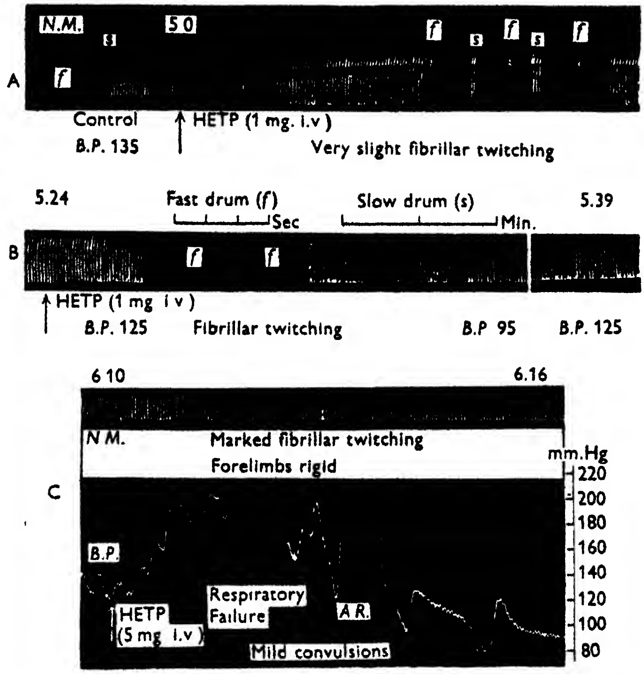


Fig. 1. Cat. 2·3 kg. Chloralose. A, B. Isometric myogram of quadriceps muscle, in response to femoral nerve stimulation at rate of 1 in 2 sec. Records are taken on a slow drum except when labelled *f* (=fast drum). In A when the drum was alternately run fast and slow, the slow records are labelled *s*. Time scales for fast drum and slow drum shown in B. C. Records from above downwards: contraction of quadriceps (as in A, B); arterial blood pressure (*B.P.*). Respiratory failure developed in C and at *A.R.* artificial respiration was applied. Injections of HETP given as signalled by arrows. (See text.)

Electrical studies show that the increase in the tension, and the delayed relaxation of the muscular response to a single stimulus applied to the motor nerve, are due to repetitive asynchronous firing of the muscle fibres. This response can be attributed to the persistence of the acetylcholine transmitter at the motor end-plates owing to the anti-cholinesterase action of the condensed alkyl phosphates. In other words, the muscle response to a single nerve stimulus is now not a twitch but an irregular diminishing tetanus. Close intra-arterial injection of acetyl choline likewise produces not a twitch but a brief asynchronous tetanus (Brown, Dale & Feldberg, 1936; Brown, 1937*a*).

With belly-tendon leads (hypodermic needles introduced into the muscle) the muscle action potential recorded by us in response to a single maximal nerve volley resembled the somewhat irregular diphasic wave, sometimes of complex character, obtained by Eccles & O'Connor (1939) from the soleus and anterior tibial muscles of the cat. The degree of complexity of the diphasic wave can be modified by adjustment of the position of the belly lead, and we have always moved the needle so as to give as simple a diphasic wave pattern as possible. Under these latter conditions the total duration of the action potential is 15–20 msec. We shall call the two phases of the 'elementary'

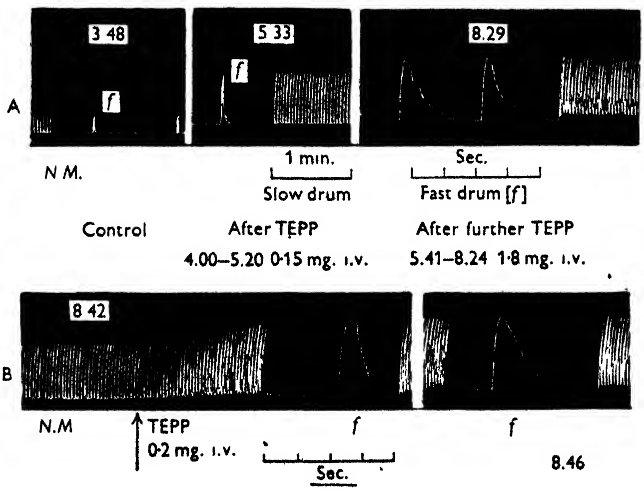


Fig. 2. Cat. 2·3 kg. Chloralose. A, B. Isometric myogram of quadriceps muscle in response to stimulation of femoral nerve at rate of 1 in 2 sec. Records on slow drum unless indicated by *f* (=fast drum); time scales in A and B. Effect of intravenous injection of TEPP. (See text.)

diphasic wave the first and second phase respectively. It is not always possible, however, to find a position of the electrode in which the whole action potential is complete in less than 20 msec., and the return stroke of the second phase of the action potential wave may be prolonged.

The first changes observed in the muscle action potential produced by HETP and TEPP are successively: (i) prolongation of the descending limb of the second phase; (ii) appearance of slight irregularities in this prolonged limb; (iii) these irregularities gradually become larger and take the form of repetitive waves; (iv) at this stage the descending limb of the second phase (of the initial diphasic wave) descends more rapidly so that the total duration of the initial diphasic variation may be shorter than in the control records.

In Fig. 3 are seen the muscle (belly-tendon) action potentials set up in response to a single maximal motor nerve volley in an experiment in which the total dose of HETP used was 20 mg. (intravenously). The first two sweeps at 12.50 and

3.57 p.m. show almost identical control records consisting only of a slightly irregular diphasic wave. After the injection of 5 mg. of HETP during 20 min. the electrical response (4.13 p.m.) showed what might be called 'peripheral afterdischarge', i.e. small high-frequency waves following on an initial diphasic wave which may be of control magnitude and duration or slightly shortened in duration as mentioned above; the peripheral afterdischarge lasted for about 150 msec. At 5.59 p.m., the initial diphasic response is followed by two larger

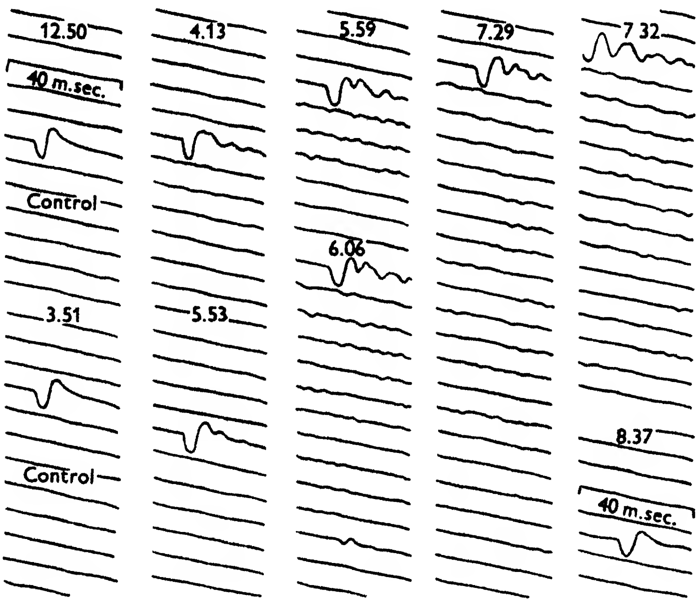


Fig. 3. Cat. 4·3 kg. Chloralose. Action potentials (belly-tendon leads) of quadriceps muscle in response to femoral nerve stimulation at rate 1 in 10 sec., showing successive stages in the development of peripheral afterdischarge, and its decline, due to the action of HETP. Each record reads from above downwards and from left to right. One sweep = 40 msec. 12.50 and 3.51 p.m., controls. HETP was then given as follows: 3.53–4.13 p.m., 5 mg. by intravenous infusion; 4.30 p.m., 1 mg. i.v.; 5.54 p.m., 1 mg. i.v.; 6.03 p.m., 1 mg. i.v.; 7.28 p.m., 1 mg. i.v.; 7.53–8.35 p.m., 11 mg. i.v. in divided doses. The figures heading each record are the times at which the records were taken. (See text.)

waves doubtless representing the synchronous firing of many muscle fibres. A further 1 mg. of HETP was injected at 6.03 p.m.; the maximum effect of this dose appeared at 6.06 p.m. The two secondary waves were now almost as large as the initial diphasic response; the small wave discharge lasted about 500 msec. Towards the end of the response silent periods became more numerous and longer in duration. The discharge thus develops a very irregular appearance. At 7.29 p.m. after another dose of 1 mg. the repetitive electrical response persisted for about 800 msec. By 7.32 p.m. the duration of the response was beginning to decline. Further doses totalling 8·5 mg. produced progressive

extinction of the peripheral afterdischarge and at 8.37 p.m. the action potential had been reduced to a simple diphasic wave without repetition and with an amplitude only 80% of the original control responses.

In Fig. 4 the mechanical and electrical responses are compared before and after the injection of 2·5 mg. of HETP over 80 min. in the decerebrate cat. The prolongation of relaxation in the mechanical record is evidently due to the repetitive firing of the muscle fibres which is clearly seen in the electrical record.

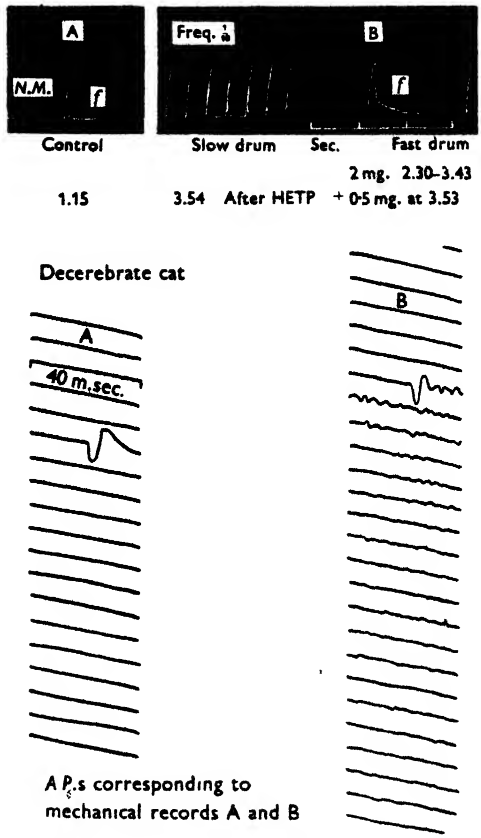


Fig. 4. Cat. 3·8 kg. Decerebrate. Upper records: isometric myogram of quadriceps (*N.M.*) in response to femoral nerve stimulation at rate 1 in 10 sec. Lower records: action potentials (*A.P.*) of same muscle—using belly-tendon leads. A, controls; B, showing effects of intravenous injection of 2·5 mg. HETP in divided doses. (See text.)

We should mention that in conformity with the results of Eccles & O'Connor (1939) we have sometimes observed in the decerebrate cat (but not under chloralose anaesthesia) repetitive action potentials in response to single maximal nerve volleys as a variation of the normal pattern. The repetition

produced by the drugs is, however, more marked and more prolonged than that ever noted in untreated decerebrated animals.

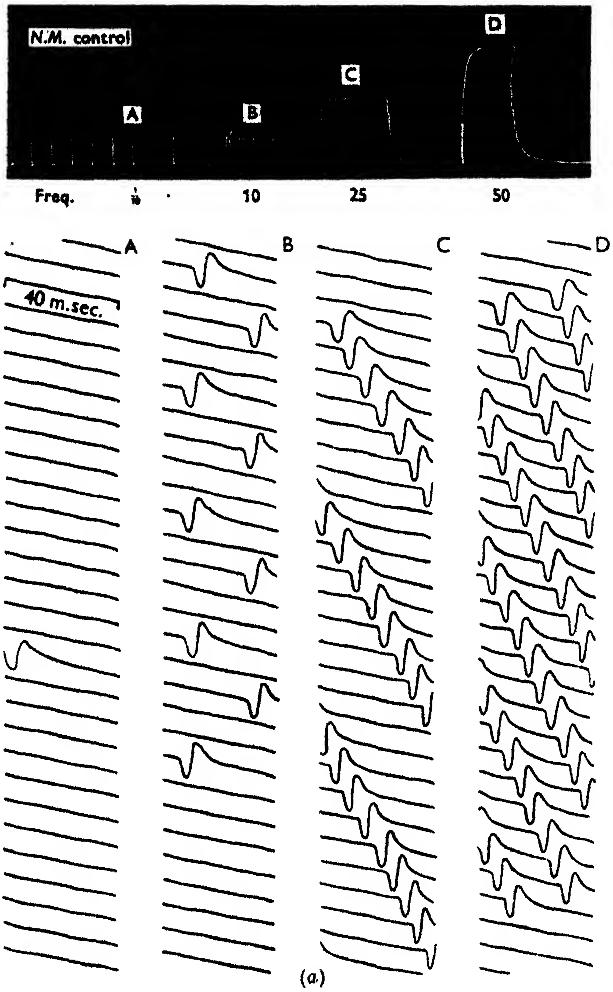
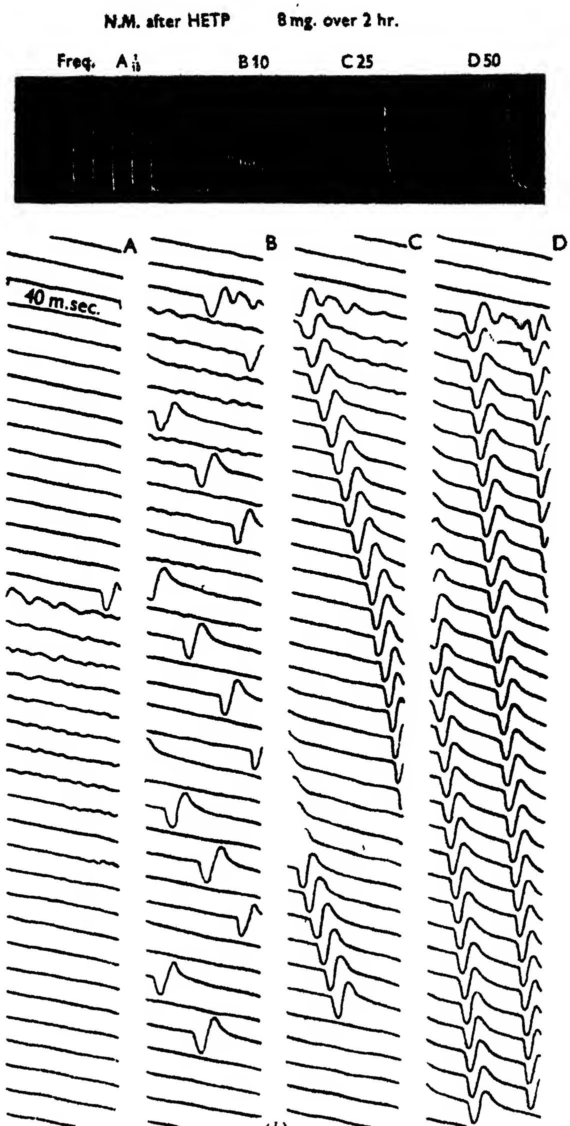


Fig. 5. Cat. 4·3 kg. Chloralose. Upper records: isometric myogram of quadriceps (*N.M.*) in response to femoral nerve stimulation at nominal rates: A, 1 in 10 sec.; B, 10; C, 25; and D, 50 per sec. Lower records: corresponding muscle action potentials. (*a*) Controls. (*b*) (See p. 382.) Showing effects of HETP after the following doses: 3.53–4.13 p.m., 5 mg. by intravenous infusion; 4.30 p.m., 1 mg. i.v.; 5.54 p.m., 1 mg. i.v.; 6.03 p.m., 1 mg. i.v. Records taken at 6.07 p.m. (See text.)

Response to supramaximal repetitive motor-nerve stimulation. The muscle responses to repetitive supramaximal stimuli at frequencies ranging from 1 in 10 sec. to 50 per sec. were studied. Fig. 5*a* shows a control series and Fig. 5*b* the responses of the muscle after the administration of 8 mg. of HETP over

a period of 2 hr. The mechanical response to stimulation at once in 10 sec. is recorded on a slow drum; the response to higher rates of stimulation (10, 25, 50 per sec.) on a fast drum.



(b)

Fig. 5. For legend see p. 381.

After HETP the mechanical responses were modified as follows. Single twitch responses (1 in 10 sec.) showed in this experiment an increase of 160% in the tension. At stimulus frequency 10 per sec. the response showed a greater

initial tension than in the control but the tension rapidly declined, as stimulation was continued, to the same level as in the control curve. At stimulus frequency 25 per sec. the initial tension was some 40% greater than in the control: it declined slightly, whereas the tension of the control rose slightly over the same period of stimulation. The final tension was 20% greater than the final tension in the control. At stimulus frequency 50 per sec. the tension was diminished by about 10% throughout. Our general experience has been that the mechanical responses to higher stimulus frequencies are less affected by HETP than those to lower frequencies. With doses of HETP which produce enhancement of the single twitch response (frequency 1 in 10 sec.) the mechanical response to frequencies 10 and 25 per sec. is at times increased and at others diminished.

The electrical records in the control experiments at all stimulus frequencies show diphasic responses which faithfully follow in frequency the rate of nerve stimulation; with the higher stimulus rates there is a slight progressive reduction in the duration of individual diphasic responses, but there is no decrease in response amplitude and no peripheral afterdischarge occurs.

After HETP the electrical responses were modified as follows. At stimulus frequency 1 in 10 sec. the muscle-action potential showed some 500 msec. of repetition like that described above. The electrical responses to the more rapid rates of stimulation show characteristically rapid extinction of the peripheral afterdischarge. Thus at stimulus frequency 10 per sec., associated with the initial potentiation of tension, the first responses show peripheral afterdischarge. In the first electrical response there are several large secondary waves after the initial diphasic response, followed by small-amplitude high-frequency waves which fill the entire stimulus interval; the large secondary waves are no longer present in the second electrical response though the smaller waves still appear; in the eighth and subsequent responses no peripheral afterdischarge is present. At the higher stimulus rates (25, 50 per sec.) a similar train of events occurred. The first electrical response shows large secondary waves only. In the second, third and fourth responses the large secondary waves are absent, but the interval is filled with small-amplitude waves. In subsequent responses the peripheral afterdischarge completely disappears and the simple diphasic responses to each stimulus closely resemble those obtained in the control period.

Anti-curari action of HETP. The anti-curari action of HETP demonstrated by Burgen *et al.* (1949) on the rat-isolated phrenic nerve-diaphragm preparation can readily be shown in cat muscle receiving its normal blood supply. In Fig. 6 supramaximal stimuli were applied to the motor nerve at a rate of 1 in 2 sec. Intocostrin (Squibb) was used for curarization and was injected intra-arterially (into the central end of the opposite iliac artery with the medial sacral artery tied) in two doses totalling 0·6 rabbit head-drop units. The mechanical response rapidly declined to a minimum representing 10% of the control tension; the

peak depression lasted about 5 min.; recovery then set in and was complete in a further 10–12 min. A second curarizing dose was given which produced the same initial depressing effect as before; 2 mg. of HETP were injected intravenously when the depression was at its maximum. Recovery set in immediately, developed rapidly and was almost complete in under 3 min.

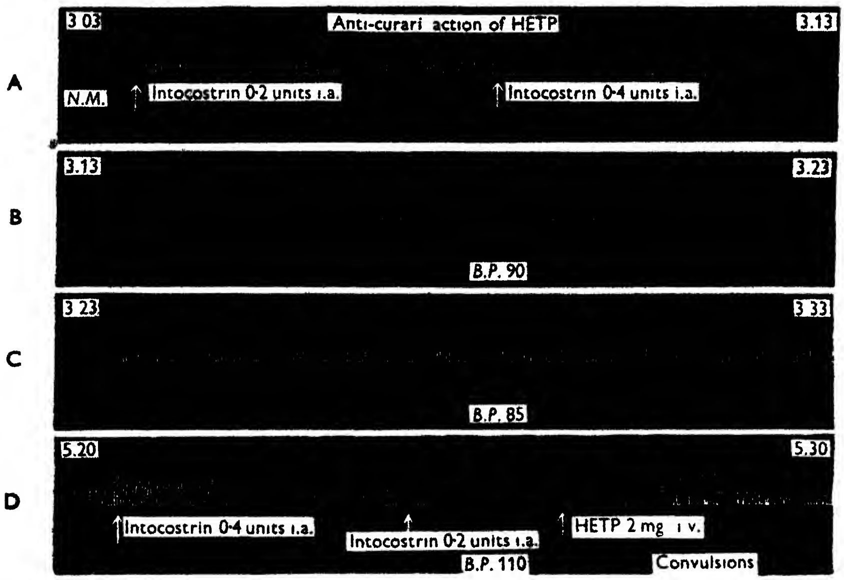


Fig. 6. Cat. 1·9 kg. Chloralose. Isometric myogram of quadriceps (*N.M.*) in response to femoral nerve stimulation at rate 1 in 2 sec. Records (A, B, C, D) read from left to right and from above downwards. Time is shown above records. In A: inject 0·2, 0·4 units of Intocostrin intra-arterially. In D: inject successively 0·4, 0·2 units of Intocostrin intra-arterially, 2 mg. of HETP intravenously. (See text.)

Action of curari on peripheral afterdischarge produced by HETP. Suitable doses of curari abolish the peripheral afterdischarge produced by HETP. Fig. 7*a* shows a control muscle action potential in response to a single maximal nerve volley. After intravenous injection of 4·75 mg. of HETP in divided doses characteristic peripheral afterdischarge, lasting about 40 msec., developed (Fig. 7*b*). One minute after an intra-arterial injection of Intocostrin (Squibb) partial extinction of the afterdischarge occurred (Fig. 7*c*). After further doses of Intocostrin the afterdischarge declined further and was finally extinguished (Fig. 7*d*, *e*), the action potential now closely resembling the control record. Partial recovery occurred as the effect of the curari wore off (Fig. 7*f*, *g*). Intravenous injection of further doses of HETP totalling 4·75 mg. caused a return and progressive increase of the peripheral afterdischarge (Fig. 7*h*, *i*). In Fig. 7*i* the afterdischarge lasted about 110 msec. It was again extinguished

by a further dose of Intocostrin (Fig. 7*j*), and the action potential once more resembled the original control. The condensed alkyl phosphates thus not only have an anti-curari action, but their action on muscle is likewise antagonized by curari.

Effect of HETP on denervated muscle. In three cats the femoral nerve on one side was cut and the animals allowed to survive for 2–5 weeks. Under chloralose anaesthesia, using supramaximal stimulation, the twitch responses of the normal quadriceps muscle, stimulated through its nerve, and of the chronically

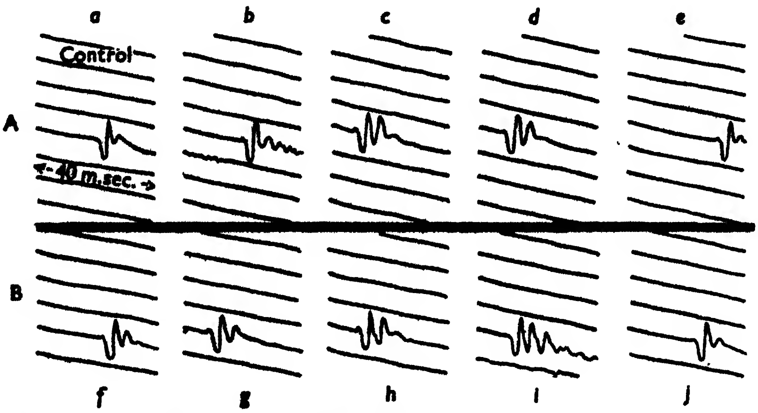


Fig. 7. Cat. 3·8 kg. Chloralose. Action potentials (belly-tendon leads) of quadriceps muscle in response to femoral nerve stimulation at rate 1 in 5 sec. A (*a*) control 4.33 p.m.; (*b*) 5.21 p.m. after injection of 4·75 mg. of HETP in divided doses; (*c*) 5.23 p.m. 1 min. after intra-arterial injection of 1 rabbit head-drop unit of Intocostrin (Squibb); (*d*) 5.25 p.m. after further dose of 0·5 unit of Intocostrin; (*e*) 5.29 p.m. after further dose of 1 unit of Intocostrin. B. (*f*) 5.42 p.m. and (*g*) 6.02 p.m. partial recovery; (*h*) 6.56 p.m. and (*i*) 7.01 p.m. after injection of 4·75 mg. of HETP in divided doses; (*j*) 7.04 p.m. after injection of 1 unit of Intocostrin. (See text.)

denervated muscle, stimulated directly, were compared before and after injection of suitable doses of HETP. The normal muscle after HETP showed characteristic peripheral afterdischarges: in the chronically denervated muscle no repetitive action potentials were observed.

Action on reflexes

Intact animal under chloralose anaesthesia. Fig. 8 shows the characteristic changes in the reflexes produced by HETP. In the experiment illustrated the flexor reflex was recorded in one hindlimb and the knee jerk in the other. After intravenous injection of 3·5 mg. of HETP in divided doses in 90 min. the flexor reflex remained unaffected, but the extensor reflexes were facilitated; the height of the knee jerk was about doubled and the crossed extensor reflex, which was initially absent, made its appearance. A further injection of 0·4 mg. produced a significant increase in the flexor reflex. The stimulus for the knee

jerk was discontinued to enable the crossed extensor in that limb to be studied in an uncomplicated manner. The crossed extensor reflex was now found to be still further enhanced and spontaneous extension movements of the limb began to appear. A further dose of 1 mg. produced more violent 'spontaneous' movements sufficiently general to merit the appellation of 'convulsions'. The 'convulsions' and the crossed extensor responses in the limb became so intermingled that they could no longer be readily differentiated. The flexor responses,

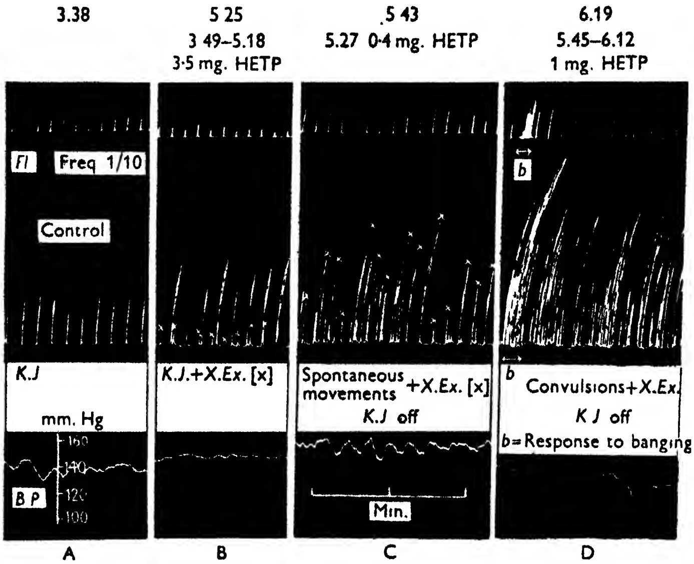


Fig. 8. Cat. 5·2 kg. Chloralose. Upper record: isometric recording of flexor reflex (*Fl.*) (anterior tibial muscle) in response to ipsilateral popliteal nerve stimulation (central end) at rate of 1 in 10 sec. Middle record: knee jerk (*K.J.*) and crossed extensor reflex (*X.Ex.*), marked with a × in B and C in response to contralateral popliteal stimulation at 1 in 10 sec. Lower record: blood pressure (*B.P.*). The knee jerk was not elicited in C and D. The records show the potentiating action of HETP on *Fl.* and *K.J.* responses and on genesis of *X.Ex.* response, responses to table banging (during period marked *b*) and spontaneous activity. (See text.)

unlike the extensor, showed little further potentiation. The stimulus of table-banging, which was without effect during the control period, produced at this stage regular reflex responses not less notably in the flexor muscles than in the extensor.

As the level of arterial blood pressure changed very little throughout the experiment the reflex changes described are entirely independent of alterations in the circulation.

Small doses of the drug have been noted to stimulate respiration and larger doses to cause respiratory failure. This, when remedied by artificial respiration, was not accompanied by circulatory collapse. From previous work (Schweitzer & Wright, 1937*a*) it is clear that these changes in breathing do not affect

somatic reflexes. Terminally, the blood pressure may fall to a very low level; the reflexes then tend to decline or disappear as a secondary effect.

The question must be carefully considered as to what extent the changes recorded in the reflexes are due to the demonstrated changes in the nerve-muscle responses and to what extent to an action on the central nervous system. It will be proved later, using the techniqué of intrathecal injection, that the condensed alkyl phosphates have a direct stimulating action on the nerve centres (spinal cord). But at this stage some suggestive evidence may be mentioned: (1) there is no quantitative relationship between the magnitude of the potentiation of the nerve-muscle response and of the reflexes. (2) The effect

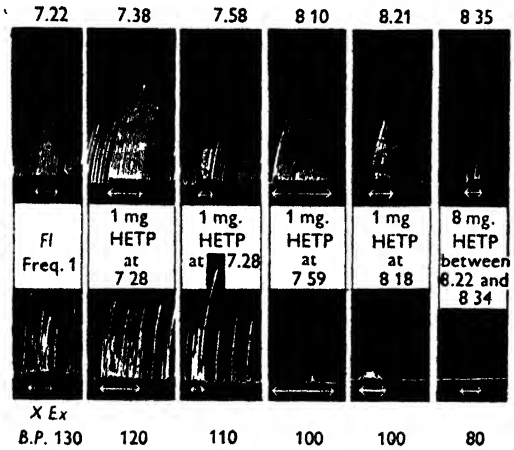


Fig. 9. Cat. 4·3 kg. Chloralose. Upper record: isometric recording of flexor reflex (*Fl.*) (anterior tibial muscle) in response to ipsilateral popliteal nerve stimulation (central end) at rate of 1 per sec. Lower record: crossed extensor response (*X.Ex.*) to same stimulation. Period of stimulation indicated by double arrow heads. Blood pressure (*B.P.*) in mm. Hg. The records show effect of HETP on these reflexes and on the background of spontaneous activity initially present. (See text.)

on the nerve muscle and on the reflexes do not necessarily follow the same temporal course; e.g. after the nerve-muscle potentiation has reached its peak the reflex responses may go on growing in size, or depression of one may coincide with enhancement of the other. (3) The changes in different reflexes are not quantitatively similar (Fig. 8). (4) The appearance of reflexes that were initially absent, and especially the onset of 'spontaneous' convulsions, can only be due to central effects of the drug.

Fig. 9 illustrates some further points. The animal had received intravenously 8 mg. of HETP in divided doses over a period of 2 hr. One hour had elapsed after the last injection before the first record in this figure was taken. The record opens with a background of fairly regular 'spontaneous' contractions

in both flexor and extensor muscles. Stimulation of the central end of the popliteal nerve at a frequency of 1 per sec. elicited an ipsilateral flexor reflex and a contralateral extensor reflex. The initial and the immediately following flexor response in any one series of stimuli were considerably larger than the later responses. Another 1 mg. of HETP was injected intravenously; 10 min. later the 'spontaneous' movements in the flexor muscles were enhanced; the flexor reflex elicited by ipsilateral popliteal nerve stimulation was markedly increased (by 100%) both as regards the large initial contractions and the succeeding somewhat smaller reactions. The crossed extensor reflex showed a smaller increase. Further injections of HETP led to depression of all the reactions, the 'spontaneous' movements and crossed extensor reflex disappearing first; at this stage the flexor reflex began to decline. It was almost completely abolished after further injections totalling 9 mg. In this experiment the arterial blood pressure fell from over 130 to 80 mm. Hg, but the latter level is of course quite adequate to maintain normal reflex reactions.

In Fig. 10 the effect of TEPP on a number of reflexes was studied simultaneously, namely reflex responses of peroneus longus (an evertor) and tibialis anticus (a flexor), elicited by stimulation of the central end of the ipsilateral nerve, and the crossed extensor reflex of the quadriceps of the opposite side. The effects of different frequencies of afferent stimulation were specially examined. The insets *a*, *b*, *c*, in Fig. 10 show the reflex responses of the muscles mentioned to short periods of stimulation at frequencies of 1 in 2 sec. and 1, 2, 4, 10, 25 and 50 per sec.; peroneus longus gave small responses at all stimulus frequencies up to 25 per sec., reflex fusion occurring at 25 per sec.; there was no response at 50 per sec. Tibialis anticus responded somewhat differently; a contraction followed each stimulus at 1, 2 and 4 per sec., there was a single initial contraction in response to 10 per sec., the succeeding volleys arousing no reaction, and there was no response to the higher stimulus frequencies of 25 and 50 per sec. In chloralosed cats we have frequently noted this lack of response or rapid dying down of the response in the flexor reflex at these stimulus frequencies. The control crossed extensor reflex only responded to stimulation at 4 and 10 per sec.; at 4 per sec. the response was tremulous, at 10 per sec. there was a small initial response only. Less than 1 min. after an intravenous injection of 0·05 mg. of TEPP the crossed extensor reflex, initially absent to stimulation at 1 in 2 sec., made its appearance and progressively increased in amplitude. Extensor tone also increased. In the case of the peroneus longus reflex, at each frequency at which responses had previously been elicited, larger responses were obtained; stimulus frequency 50 per sec. which was previously ineffective produced a tetanic response maintained somewhat below the initial peak value. With the tibialis anticus reflex, the previously ineffective rates of 25 and 50 per sec. now produced responses and the response to 10 per sec. was repetitive instead of initial only. The changes were most dramatic with the

crossed extensor reflex. Stimulus frequency of 1 in 2 sec. which in the control period was ineffective now gave large regular responses to each volley, the peak tension rising as stimulation was continued, representing recruitment; relaxation was incomplete between the volleys, representing central afterdischarge;

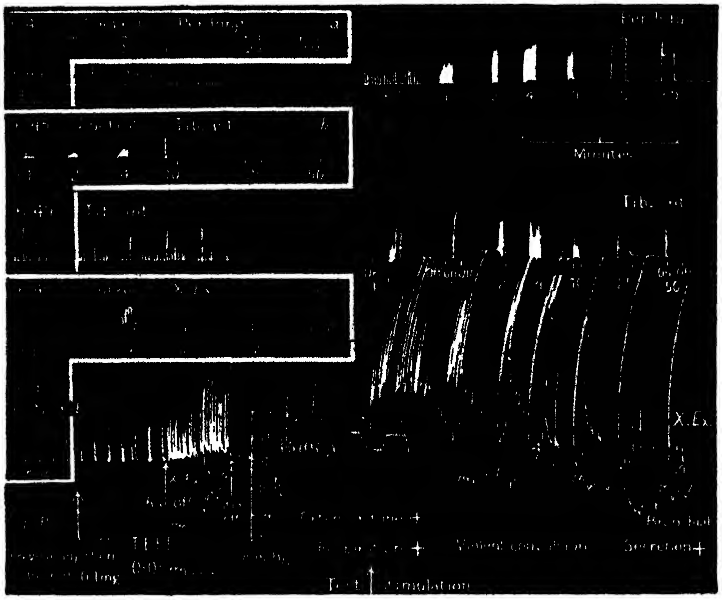


Fig. 10. Cat. 3·7 kg. Chloralose. Inset *a*, *b*, *c* are control responses: *a* of m. peroneus longus (per. long.); *b* of m. tibialis anticus (tib.ant.) to ipsilateral, and *c* of m. quadriceps (crossed extensor reflex-*X.Ex.*) to contralateral, popliteal nerve stimulation at rates of 1, 2, 4, 10, 25, 50 per sec. (F 1–F 50) and 1 in 2 sec. (F ½). The lower part of inset *c* shows the knee jerk elicited by stimulating at 1 in 12 sec., as well as absence of *X.Ex.* to F ½. The main records from above downwards: response of m. peroneus longus; of m. tibialis anticus; of m. quadriceps; blood pressure (*B.P.*). At arrow inject 0·05 mg. of TEPP intravenously (after previous injection of 0·1 mg.). When the crossed extensor reflex began to appear on the quadriceps record the knee jerk hammer was switched off (second arrow). The quadriceps record now shows the crossed extensor reflex only. At third arrow all nerve stimulation was temporarily stopped and the drum was run for 2 min. At arrow labelled 'Test stimulation' the responses of m. peroneus longus, m. tibialis anticus and m. quadriceps were tested to bursts of stimuli (duration indicated by 'on', 'off') at 1 in 2 sec., 1, 2, 4, 10, 25 and 50 sec. (See text.)

towards the end of the bout of stimulation the responses declined, but some central afterdischarge was still noticeable on cessation of stimulation. At 1 per sec., relaxation between the volleys was even less complete and there was considerable central afterdischarge. At 2 and 4 per sec. more complete reflex fusion was occurring, while at 10 per sec., reflex fusion was complete at a level somewhat below the opening contraction. Stimuli of 25 and 50 per sec. only produced initial responses.

It is interesting to note that the detailed changes in the two ipsilateral reflexes studied were not identical, illustrating the danger of generalizing about the central action of these drugs from a study of too few reflex types. The crossed extensor reflex brings out well some features not so well shown by the flexors, namely, how the drug facilitates recruitment and central afterdischarge, as well as facilitating responses previously not elicitable. Presumably reflex fields previously subliminally stimulated are brought up to discharge level of central excitation. The results described were accompanied by a rise and subsequent decline of blood pressure and stimulation of breathing, but not to an extent that might be expected to modify the reflex reactions significantly (Schweitzer & Wright, 1937*a*).

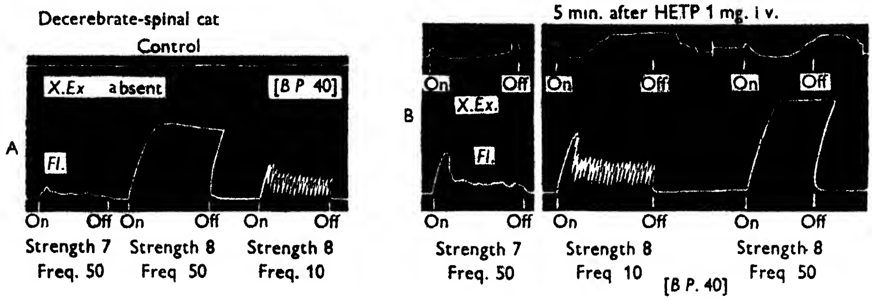


Fig. 11. Cat. 3·0 kg. Decerebrate-spinal. A, B. Lower record: isometric recording of flexor reflex (*Fl.*) (anterior tibial muscle) in response to ipsilateral popliteal nerve stimulation. Upper record: crossed extensor response (*X.Ex.*) to same stimulation. A, control records—three bursts of stimulation were employed at strengths (arbitrary units) and frequencies indicated. B, action of HETP in potentiating flexor and facilitating crossed extensor responses. (See text.)

Decerebrate animal made spinal. To eliminate the action of the chloralose anaesthesia and the influence of supraspinal levels, studies were carried out in decerebrate animals made spinal. In the experiment illustrated in Fig. 11 stimulation was carried out at various strengths (expressed in arbitrary units) and frequencies. The crossed extensor reflex could not be elicited at any of the strengths or frequencies employed. The flexor reflex showed a feeble irregular response at strength 7, frequency 50; a powerful well-sustained tetanus at strength 8, frequency 50; and a weaker repetitive response to stimulation at strength 8, frequency 10. Five minutes after the injection of 1 mg. of HETP the crossed extensor reflex appeared in a complex form with a well-marked afterdischarge. Both responses at strength 8 showed evidence of an inhibitory component in the afferent nerve which was gradually overcome by afferent excitation. The potentiation of the flexor reflex was not so dramatic, but at strength 7, frequency 50, the opening reflex contraction was markedly increased and it was better sustained; at strength 8, frequency 10, the opening response was doubled; and at strength 8, frequency 50, it was increased by 15%.

Intrathecal injection of alkyl phosphates

Conclusive evidence about the central action of the alkyl phosphates is provided by experiments in which injections were made intrathecally (Calma & Wright, 1947) generally at the level of the seventh lumbar vertebra. In the experiment illustrated by Figs. 12 and 13 mechanical and electrical records* of the nerve-muscle responses showed no change whatever, demonstrating that the drug had not entered the general circulation in significant doses. The blood pressure and respiration likewise remained unaltered till nearly the end of the experiment. The reflexes studied were the knee jerk, flexor reflex and crossed extensor reflex, the two latter in response to bursts of repetitive stimulation at frequencies of 1 in 10 sec. and 1, 2, 5, 10, 25, 50 and 100 per sec. The controls (Fig. 12*a*) showed very small flexor responses at stimulus frequency of 1 and 2 per sec.; fused responses (sometimes with rising tension) at 5 and 10 per sec.; a fused response rising rapidly to a peak and then declining, at 25 per sec.; a similar initial reaction but with rapid decline of tension almost to zero in spite of persistent stimulation, at 50 and 100 per sec. The crossed extensor could not be elicited. Control intrathecal injections of propylene glycol gave the following results: 0·1 c.c. of 1% propylene glycol in saline was without effect; 0·1 c.c. of 10% propylene glycol in saline gave a sharp rise of blood pressure which declined slowly and a very transient increase in the knee jerk; 0·1 c.c. of 100% propylene glycol, which produced a bigger and more rapid rise of blood pressure with a still slower recovery, temporarily depressed the knee jerk on the first injection and produced transient extensor spasm after the second injection. There were no persistent effects. The doses of TEPP used were such that, on dilution with saline from a 1% solution in propylene glycol, the vehicle for injection was a solution of propylene glycol ranging from 1 to 10%, usually 5%. It can be safely concluded that the changes in the reflexes, described below as following the intrathecal injection of TEPP, are due to the drug and not to the vehicle.

Intrathecal injection of 0·01 mg. of TEPP produced a transient rise of blood pressure but no changes in the somatic reflexes. Repeated injections of 0·05 mg. of TEPP amounting in all to 0·4 mg. over 135 min. produced the changes illustrated in Figs. 12 and 13.

Knee jerk (Fig. 13*a*). There is a progressive increase in the tension of the response, delayed relaxation and the development of secondary contractions. The increased tension can be attributed to central facilitation, the latter changes to enhanced and prolonged afterdischarge.

Flexor reflex. The initial change was an increased response to stimulus frequencies 1 and 2 per sec. (Fig. 12*b*). Later the level or descending plateau

* The action potentials were recorded with both high and low amplification, the former giving overloading on the initial diphasic response, but enabling easier detection of the onset of repetition.

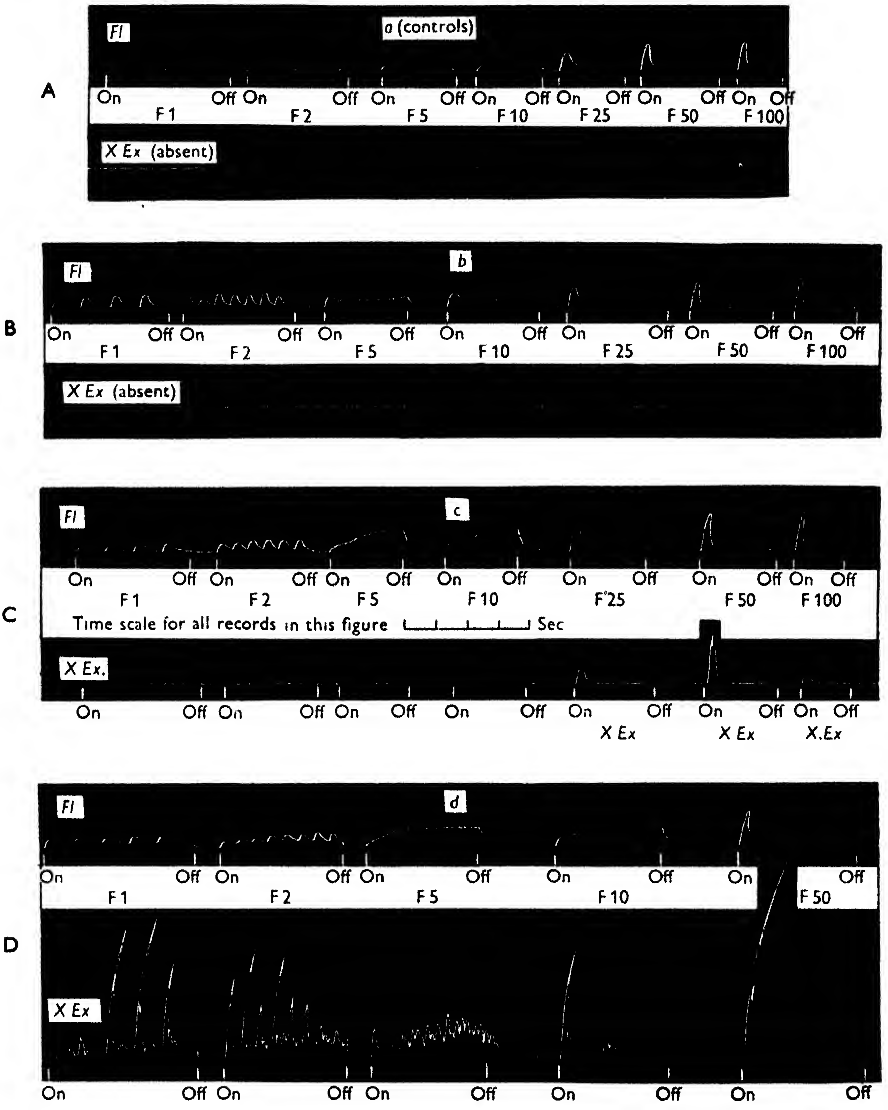


Fig. 12. Cat. 2·8 kg. Chloralose. A, B, C, D. Upper record: flexor reflex (*Fl.*) (anterior tibial muscle) to ipsilateral stimulation; lower record: crossed extensor reflex (*X.Ex.*) of quadriceps muscle to contralateral stimulation, of central popliteal nerve at rates of 1, 2, 5, 10, 25, 50 and 100 per sec. for periods indicated by 'on', 'off'. A. Control records—crossed extensor response absent. B. 6.57 p.m. after total of 0·12 mg. of TEPP injected *intrathecally* in divided doses. Crossed extensor reflex still absent. C. 7.52 p.m. after further 0·1 mg. of TEPP *intrathecally* in two doses. D. 8.40 p.m. after further 0·2 mg. of TEPP *intrathecally* in divided doses. (See text.)

of the fused response to stimulus frequencies 5 and 10 per sec. was converted into a smoothly or irregularly rising tension attaining a greater maximum than previously. The response to 25 per sec. showed a slightly enhanced opening contraction which was somewhat better sustained throughout the period of afferent stimulation than before (Fig. 12*c*). The responses to higher frequencies were slightly increased but otherwise unaltered.

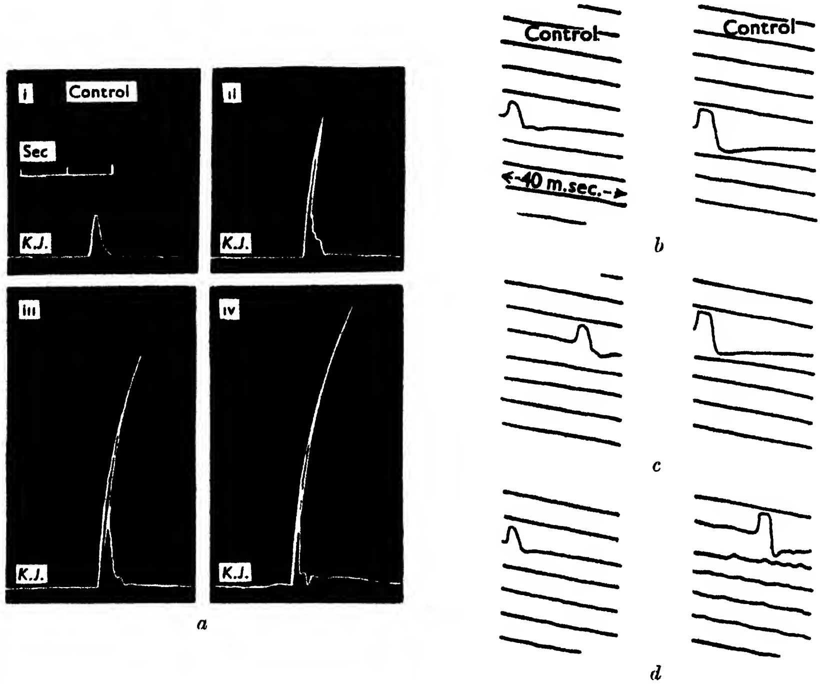


Fig. 13. Cat. 2·8 kg. Chloralose (same experiment as in Fig. 12) *a*, i–iv knee jerk (*K.J.*) on fast drum, showing potentiation with successive *intrathecal* injections of TEPP. i, control; ii, 6.55 p.m., after four injections of TEPP totalling 0·12 mg. between 6.30 and 6.51 p.m.; iii, 7.51 p.m. after two further injections of 0·05 mg. of TEPP; iv, 9.04 p.m. after six injections totalling 0·4 mg. *b*, *c* and *d* are action potentials (belly-tendon leads) of quadriceps muscle in response to femoral nerve stimulation at rate 1 in 5 sec. Left-hand record in each case is at low amplification and right-hand record at high amplification. *b*, is control. *c*, is record taken at 10.0 p.m. at peak of reflex potentiation when convulsions had set in, showing no 'peripheral afterdischarge'. *d*, is record taken at 10.10 p.m. after an *intravenous* injection of 4 mg. TEPP, peripheral afterdischarge now developing. (See text.)

Crossed extensor reflex. This reflex which was initially absent began to appear 60 min. after the first injection. At this time (Fig. 12*c*) there was a response to stimulus frequency 25 per sec. but no reaction to lower frequencies. There was a bigger response to stimulus frequency 50 per sec. and a very small one to 100 per sec. The responses consisted of 'opening' contractions dying down rapidly in spite of persistence of afferent stimulation. Fig. 12*d* illustrates the

reactions near the height of the TEPP effect. At stimulus frequency of 1 per sec., each succeeding stimulus produced for a time contractions of increasing magnitude. The relaxation curve of each reflex response was slow and irregular and displayed secondary contractions like those seen on the knee jerk. There was clear evidence of the onset of recruitment and of marked central after-discharge. With frequencies of 10 and 50 per sec. there were extremely powerful 'opening' contractions which declined irregularly and with varying speed to base-line in spite of sustained stimulation.

As the reflex changes described occurred without any alteration in the mechanical and electrical responses of the nerve muscle preparation (Fig. 13*b, c*) or changes in blood pressure or respiration, they must be due to a direct action of TEPP on the central nervous system.

Finally, 'spontaneous' movements appeared, and after a further 0·2 mg. of TEPP intrathecally they built up slowly to convulsions f increasing violence. As there was still no change in the nerve-muscle response these convulsions must likewise be of central origin. An intravenous injection of TEPP at this stage produced the usual changes in the mechanical and electrical response of the nerve muscle preparation (Fig. 13*d*), and fibrillar twitching made its appearance for the first time.

Action of atropine. The observations on HETP were generally carried out after intravenous administration of atropine (0·65 mg.). The dose of atropine needed to annul the effects of stimulation of para-sympathetic nerves may depress somatic reflex reactions (Schweitzer & Wright, 1937*b*). After a dose of 0·65 mg. the central stimulating action of HETP develops quite well. Similar observations have been made in the case of the central action of eserine, prostigmine and related methyl carbamic ester anticholinesterases (Schweitzer & Wright, 1937*b*; Calma & Wright, 1944) and of diisopropylfluorophosphonate (Chennells & Wright, 1947).

DISCUSSION

The effects of the condensed alkyl phosphates on the mechanical and electrical responses of the nerve-muscle preparation closely resemble those of eserine or prostigmine (Brown 1937*a, b*). Diisopropylfluorophosphonate produces similar effects but with larger doses (Brown, Burns & Feldberg, 1947). As with eserine the potentiating effect of the condensed alkyl phosphates is abolished after chronic denervation indicating that its action depends on the integrity of the end-plate mechanisms.

The *central* excitant action of the condensed alkyl phosphates resembles that of eserine and of diisopropylfluorophosphonate. In a number of studies (Schweitzer & Wright, 1937*b–d*, 1938; Schweitzer, Stedman & Wright, 1939; Calma & Wright, 1944, 1947; Kremer, Pearson & Wright, 1937; Kremer, 1942) it has been shown that the carbamic ester group of anticholinesterases can be divided into two classes with respect to their central action: eserine and other

tertiary compounds (e.g. methyl carbamic ester of hordenine hydrochloride) are central excitants, while the corresponding quaternary compounds like prostigmine and the methyl carbamic ester of hordenine methiodide are central depressants. Schweitzer *et al.* (1939) drew attention to the difference in the physical properties of these two classes of compounds, the convulsant tertiary compounds giving rise to lipoid-soluble derivatives, while the quaternary compounds give rise to water-soluble but lipoid-insoluble derivatives. It was suggested that these differences in solubility might determine the direction of the action on the central nervous system. As pointed out by Topley (1947) both HETP and TEPP are lipoid soluble. When HETP is dissolved in water it immediately decomposes into simpler products which are presumably the pharmacologically active principles. These decomposition products are water soluble but *lipoid-insoluble.* It seems, therefore, that with the condensed alkyl phosphates, differences in lipoid solubility do *not* modify the direction of the action on the central nervous system, both TEPP and the active products of HETP being convulsants.

Earlier studies (Schweitzer *et al.* 1939) on eserine and prostigmine showed that the central excitant action of the former and the central depressant action of the latter could be attributed to their specific anticholinesterase action. The most suggestive evidence was the fact that the central convulsant action of the tertiary group and the central depressant action of the quaternary group increased directly with their anticholinesterase activity. Furthermore, when the carbamic ester grouping was removed from the tertiary anticholinesterases both their anticholinesterase action and their central excitant action were abolished. It is natural to argue that as the condensed alkyl phosphates are convulsants and are also anticholinesterases, and as certain anticholinesterases are convulsants, that the convulsant action of the alkyl phosphates is due to their anticholinesterase activity. But too little is known for certain about the humoral mechanisms, if any, involved in central transmission to make the above argument more than merely very plausible. It would be necessary to study many other members of the alkyl phosphate series to determine whether or not the central excitant and the anticholinesterase action follow a parallel course before the two actions could be correlated as effect and cause. It should be pointed out, however, that the central excitant action of HETP and TEPP is roughly directly proportional to their anticholinesterase action, TEPP being a more powerful anticholinesterase and a more potent convulsant.

From the evidence available there is no reason to doubt that the peripheral neuro-muscular effects of the condensed alkyl phosphates are attributable to their anticholinesterase action.

SUMMARY

1. HETP and TEPP potentiate the mechanical response of the nerve-muscle preparation in the cat to single motor volleys. Using belly-tendon leads, the electrical response is characteristically modified by the addition, after the initial diphasic wave, of further waves referred to by us as 'peripheral afterdischarge' which may be repeated for a period up to 1 sec. The electrical changes are described in detail.

2. The effects of HETP and TEPP on the response to repetitive stimulation at frequencies of 10–50 per sec. are more complex. The outstanding feature of the electrical response is the disappearance of the peripheral afterdischarge after the first few responses.

3. HETP and TEPP antagonize the peripheral action of curari on the nerve-muscle preparation. Curari, injected after potentiation has been produced by HETP and TEPP, rapidly extinguishes the peripheral afterdischarge to single motor-nerve volleys.

4. The characteristic effects of HETP and TEPP on the nerve-muscle response are abolished after chronic motor denervation of the muscle.

5. HETP and TEPP facilitate various reflexes (knee jerk; flexor, peroneus, crossed extensor and jar reflex) and induce convulsions. Central afterdischarge is enhanced and prolonged. The effects described are independent of changes in the nerve-muscle response or in circulation and respiration, and are due to a direct action on the central nervous system. The central actions are obtained on intravenous and intrathecal injection in animals under chloralose anaesthesia or decerebrated, and after spinal section.

6. Attention is drawn to the similarity in the central action of the condensed alkyl phosphates to that of eserine and the tertiary carbamic ester anticholinesterases, and to that of diisopropylfluorophosphonate.

7. The peripheral neuromuscular action of HETP and TEPP can be confidently attributed to their anticholinesterase action. The mechanism of the central excitant action cannot yet be ascribed with certainty to this property of the drugs.

We are indebted to the University of London Central Research Fund for a grant to one of us (W. F. F.), and to Messrs Pal Chemicals and Messrs Albright & Wilson for supply of materials. We are indebted to Dr A. S. V. Burgen for preparation of samples of TEPP.

REFERENCES

Brown, G. L. (1937*a*). *J. Physiol.* **89**, 220.

Brown, G. L. (1937*b*). *J. Physiol.* **89**, 438.

Brown, G. L., Dale, H. H. & Feldberg, W. (1936). *J. Physiol.* **87**, 394.

Brown, G. L., Burns, B. Delisle & Feldberg, W. (1947). *J. Physiol.* **106**, 36*P*.

Burgen, A. S. V., Keele, C. A., Chennells, Mary, del Castillo, J., Floyd, W. F., Slome, D. & Wright, Samson (1947). *Nature, Lond.*, **160**, 760.

Burgen, A. S. V., Keele, C. A. & Slome, D. (1949). *J. Pharmacol.* (In the Press.)
Calma, I. & Wright, Samson (1944). *J. Physiol.* **103**, 93.
Calma, I. & Wright, Samson (1947). *J. Physiol.* **106**, 80.
Chennells, Mary & Wright, Samson (1947). *Nature, Lond.*, **160**, 503.
Eccles, J. C. & O'Connor, W. J. (1939). *J. Physiol.* **97**, 44.
Hall, S. A. & Jacobson, M. (1948). *Industr. Engng Chem.* **40**, 694.
Kremer, M. (1942). *Quart. J. exp. Physiol.* **31**, 337.
Kremer, M., Pearson, H. E. S. & Wright, Samson (1937). *J. Physiol.* **89**, 21 *P*.
Schweitzer, A., Stedman, E. & Wright, Samson (1939). *J. Physiol.* **96**, 302.
Schweitzer, A. & Wright, Samson (1937*a*). *J. Physiol.* **88**, 459.
Schweitzer, A. & Wright, Samson (1937*b*). *J. Physiol.* **89**, 165.
Schweitzer, A. & Wright, Samson (1937*c*). *J. Physiol.* **89**, 384.
Schweitzer, A. & Wright, Samson (1937*d*). *J. Physiol.* **90**, 310.
Schweitzer, A. & Wright, Samson (1938). *J. Physiol.* **92**, 422.
Topley, B. (1947). Communication to the Physiological Society (December).
Wilson, A. T. & Wright, Samson (1936–7). *Quart. J. exp. Physiol.* **26**, 127.

J. Physiol. (1949) 108, 398–404 531.732.612.27.015

METHOD FOR THE FREQUENT ESTIMATION OF FOREARM BLOOD FLOW UNDER CONDITIONS OF DECREASED ATMOSPHERIC PRESSURE

By D. McK. KERSLAKE

From the Royal Air Force Institute of Aviation Medicine, Farnborough

(*Received* 4 *June* 1948)

For the measurement of forearm blood flow at ground level the plethysmographic method of Lewis & Grant (1925) has been widely used. Under these conditions it is convenient to transmit the changes of forearm volume from the plethysmograph to some form of volume recorder by means of tubing containing air. The various recorders which have been used, and the details of technique, have been critically reviewed by Abramson (1944).

The use of air transmission when experiments are to be performed in the decompression chamber is impracticable for two reasons. First, any changes in atmospheric pressure which may occur produce fluctuations in the total volume of the closed air system and therefore in the base-line of the record. Secondly, it is clear that work must be done on the volume recorder if it is to move, and this can only be accomplished by increasing the pressure in the air system leading to it. The change of pressure necessary to operate a well-constructed mechanical recorder is so small that at ground level the volume change required to produce it is negligible. However, when the air in the system is rarefied the volume change corresponding to the same absolute increase in pressure becomes appreciable, so that the recorder lags behind the forearm volume changes, and reliable results cannot be obtained.

A method employing water-filled tubes to transmit the volume changes was described by McMichael & Snyder (1941). Volume changes were recorded by photographing the meniscus of water in a horizontal glass tube attached to the water-filled plethysmograph. Difficulties of air transmission are overcome in this way, but the method has certain disadvantages. It is difficult to ensure that no air bubbles are present in the tubing, and the base-line fluctuations due to vascular changes and to the inflation of the cuffs necessitate continuous attention and readjustment. Furthermore, in order to drive water through the narrow tubing a pressure must be built up behind it, and since the end dams of the plethysmograph cannot be made completely rigid the recorded volume

changes do not represent those actually occurring in the forearm. The error depends upon the fitting of the plethysmograph and upon the level of blood flow being recorded, and it is greatest at the beginning of the inflow curve when the greatest accuracy is required. Its extent under working conditions may not be great, but it is desirable to verify this by dynamic calibration in the case of each experiment.

METHOD

The method to be described is based upon the minimization of any form of transmission from the plethysmograph to the volume recorder. The plethysmograph is of conventional design, having a tube of uniform bore (about an inch and a quarter) fixed vertically into its upper side. When the forearm has been sealed into it (the 'sleeve' method of Grant & Pearson (1938) has been used, although other methods are equally applicable) the interior of the plethysmograph is filled with water warmed to 34° C. (Barcroft & Edholm, 1946) until the level appears in the vertical tube. As the forearm expands the water rises a little, but it is unlikely (Abramson, 1944) that this slight change in hydrostatic pressure affects the forearm blood flow. The level of water in the vertical tube is recorded directly by photographing the movements of a hollow metal float resting on its surface. As in the case of other volume recorders work must be done to move the float; in other words, the water level must rise relative to the float before the latter will move. However, if the diameter of the float is very nearly as great as that of the tube, this rise in level (which amounts to only a fraction of a millimetre under normal working conditions) can be produced by the displacement of a very small volume of water into the space between the float and the wall of the tube. Moreover, any lag resulting from this will be minimized by the pulsatile nature of the changes in forearm volume.

The float is suspended and kept clear of the wall of the tube by the simple lever system shown in Fig. 1. It will be seen that its weight is partly counterbalanced, and that this counterbalancing force is constant for all positions of the float. In this way the water displaced by the float is constant throughout the full range of movement, and the linearity of the recording system is preserved. The float does not move in a straight vertical line, but along the arc of a circle, but as its axis is at all times perpendicular to the water surface it still follows accurately the changes in water level. As the water level rises the hydrostatic pressure on the end dams of the plethysmograph increases, and a certain amount of bulging takes place. If it be assumed that the end dams are elastic, the displacement will be proportional to the pressure, and so the recorder will still be linear, although its excursion will always be less than the actual change in forearm volume. Since this effect is independent of the rate of expansion of the forearm, calibration carried out by injecting known volumes of water into the plethysmograph with the arm in place will overcome this inaccuracy.

The optical system consists of a light source and condenser focused on a cross-wire mounted above the float. A magnified image of this wire is projected by a simple lens on to the slit of a bromide paper camera. A half mirror is arranged to reflect part of the image of the cross-wire on to a viewing screen, so that the behaviour of the recorder can be watched during the experiments. The light source is interrupted to provide vertical time marks on the record.

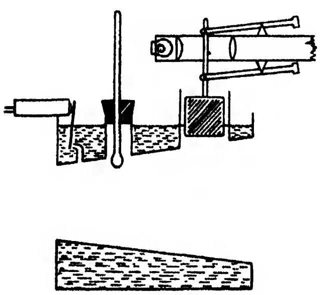


Fig. 1.

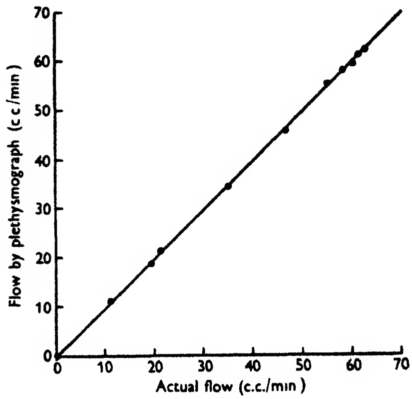


Fig. 2.

Fig. 1. Diagram of plethysmograph, showing the system of volume recording, and the arrangements for stabilizing the base-line.

Fig. 2. Dynamic calibration of volume recorder. The line drawn is a true diagonal.

The characteristics of the recording system were investigated by dynamic calibration. The ends of the plethysmograph were blocked off and a static calibration carried out by injecting measured volumes of water, recording after each the position of the cross-wire image. Water was next injected at steady-measured rates, and continuous records of the movements of the float were taken. The rates of inflow were calculated from the records obtained and the results of the static calibration. These results were plotted against the known rates of water inflow, and agreement was found to be good (Fig. 2). An example of the type of record obtained is shown in Fig. 3. It will be seen that the transition from the horizontal base-line to the sloping record when water began to enter the plethysmograph is abrupt, demonstrating that under these conditions the inertia of the system is negligible. The total inflow rate in this case was 60 c.c./min., representing a forearm blood flow of about 10 c.c./100 c.c. forearm/min.

In order to maintain constant conditions in the interior of the plethysmograph a water-bath was built round the outside, the ends being formed by extensions of the end plates of the plethysmograph. The water in the bath is kept at a fixed temperature by means of an electric immersion heater, and the

water inside the cavity of the plethysmograph is circulated through metal pipes immersed in the water-bath, by means of a small rotary pump.

With the apparatus in this form it was found that vaso-motor changes resulted in changes of resting forearm volume greater than the full-scale deflexion of the recording system. Continual adjustments had to be made by means of a syringe in order to obtain a suitably placed base-line for each inflow record. This difficulty was overcome by sealing a small tube into the wall of the plethysmograph below the level of the water in the bath. In the interval between inflow records this allowed the water-level in the vertical tube of the plethysmograph to come into equilibrium with that in the water-bath. When inflow curves were recorded this tube was blocked by a rubber pad attached to a small lever operated by a relay magnet. In this way changes in resting forearm volume are taken up over the whole area of the water-bath surface, and the base-line is remarkably constant. During readings, on the other hand, the whole of the change in forearm volume appears as a change in level of the water in the vertical tube. A considerable economy in the width of the recording paper was effected by this device, since readings could be arranged to occupy nearly the whole width of the paper. The possibility of leaks in the plethysmograph passing unnoticed because of this artificially steady base-line can be readily checked at the beginning and end of each experiment by energizing the magnet which blocks the tube, and filling the vertical tube of the plethysmograph with water. If this level does not fall it is clear that no leaks are present. In the existing model the width of the recording paper is 60 mm., representing a volume of about 6 c.c. The area of the water-bath is about forty times that of the vertical tube, so that a change in resting forearm volume of 40 c.c. will only displace the base-line by about 10 mm.

The apparatus was designed for the investigation of changes in forearm blood flow during short periods of anoxia at high altitudes. In view of the fact that the total duration of experiments was to be in the region of 3 or 4 min., it was important that as much information as possible concerning the blood flow changes in that time should be obtained. It was necessary to discover the maximum rate at which readings could be taken without interfering with the blood flow or causing inaccuracy in interpretation of the records. Preliminary work showed that it was very difficult to secure more than four readings per minute when the various pieces of apparatus were operated manually, and the dexterity of the operator appeared to be the limiting factor.

A control mechanism was therefore constructed which would carry out the various operations as frequently as might be thought desirable. This part of the apparatus is based on a uniselector driven by the time clock which also provides the time marks on the record. The selector is wired to move from one to five 'clicks' for each impulse from the clock, so that it makes one complete revolution in from ten to fifty impulses, according to the setting of a switch.

One of the contact banks, which is swept during alternate half turns of the selector, is wired via a switch to a relay, which can be arranged to close for a variable proportion up to 50% of the total duration of the cycle. Contacts on this relay operate the camera motor and lamp, close the communication

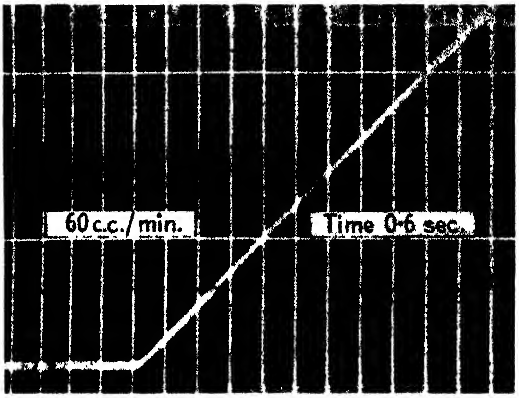


Fig. 3. Record obtained during dynamic calibration. Inflow rate 60 c.c./min.; time marker 0·6 sec.

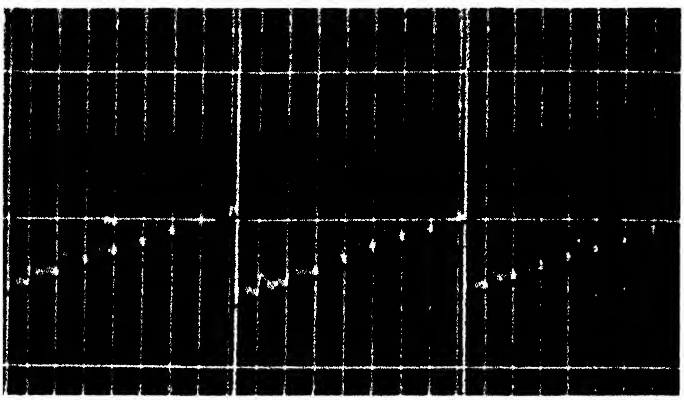


Fig. 4. Section of blood flow record obtained on a resting subject. Interval between readings 9·6 sec. Duration of each period of venous occlusion 4·8 sec. Time marker 0·6 sec. Forearm blood flow about 2 c.c./100 c.c. forearm/min.

between the plethysmograph and water-bath, and open a valve which allows the collecting cuff to be inflated. These actions can also be performed separately by manual control or simultaneously by the operation of a switch. The apparatus can be set to take up to ten readings per minute at regular intervals, and once it has been set going it operates without further interference.

Some experiments were performed in order to discover the optimum rate for taking readings. It was found that so long as the collecting pressure was not applied for more than 50% of the total time no changes in forearm blood flow could be attributed to the effect of the collecting pressure cycle. If readings are arranged to occupy this proportion of the time it is clear that the duration of each will be in inverse proportion to the frequency of the readings. It was found that if readings were taken more frequently than six times a minute it became progressively more difficult to interpret the slope of the shorter records. On the other hand, no appreciable increase in accuracy of interpretation was obtained by making the readings last longer than 5 sec. each, so that it appeared that a maximum of information about blood flow changes could be obtained by taking readings at the rate of six per minute. An example of the type of record obtained under these conditions is shown in Fig. 4.

The arrangements for the inflation of the collecting cuff are complicated by the need for the maintenance of a steady pressure in the cuff during the time of its inflation. It is almost impossible to avoid some sort of jump of the base-line at the moment of inflation. In itself this does not interfere with the reading of the record, but if the cuff pressure changes slowly during the period of recording a slow change in base-line might be expected to result. The slope of the record would still be uniform, but would be in part due to the arterial inflow to the forearm and in part due to the artefact produced in this way. It would not be possible to separate these components, so that accurate computation of blood flow would be impossible.

It was found that an ordinary reducing valve did not operate fast enough to ensure a steady cuff pressure, so that a reservoir is used from which the cuff is suddenly inflated. The reservoir is supplied from a high-pressure cylinder via a reducing valve, which is connected to it by two lines. One contains a solenoid-operated valve and an adjusting tap, the other a second adjusting tap. When the cuff is not inflated the solenoid is energized allowing air to enter the reservoir along this route. When the pressure builds up to a predetermined level the solenoid supply is cut off by contacts operated by a pressure capsule. The second inflow channel remains open, and this is adjusted to compensate for leaks, which were found to be unavoidable. When the cuff is inflated the reservoir pressure falls a few millimetres, but the main air supply remains cut off. The pressure remains at this new level as long as the collecting cuff is inflated, the leaks being compensated by the pilot air flow described above. As soon as the cuff is deflated the main air supply is again switched on, and the cycle is repeated. The valve used for the inflation of the collecting cuff is of German origin, and was originally designed for pneumatic aircraft gear. It is in effect a solenoid-operated two-way tap, and can therefore be arranged to allow the collecting cuff to deflate in between readings. The tubes are of large bore (about half an inch) so that inflation and deflation of the cuff are very rapid.

The arterial occlusion cuff round the wrist is supplied from a second reservoir fed from the same air supply through another tap. Both reservoirs can be opened to the atmosphere during ascent and descent.

SUMMARY

1. A modification is described of the plethysmographic method of Lewis & Grant (1925), suitable for use under conditions of decreased atmospheric pressure.

2. Volume recording is carried out by photographing the movements of a float resting on the surface of the water in the vertical wide-bore tube of a conventional plethysmograph.

3. The base-line is kept constant by opening a communication between the plethysmograph and water-bath in the intervals between readings.

4. The inflation of the collecting cuff and operation of the recording mechanism are performed automatically by an electric controller.

5. It was found that accurate estimations of forearm blood flow could be obtained from inflow curves lasting 5 sec., and that these could be repeated at intervals of 10 sec. without affecting the forearm blood flow.

I am grateful to the Director General of Medical Services, R.A.F., for permission to publish this paper.

REFERENCES

Abramson, D. I. (1944). *Vascular Responses in the Extremities of Man in Health and Disease*, pp. 65–7. University of Chicago Press.

Barcroft, H. & Edholm, O. G. (1946). *J. Physiol.* **104**, 366.

Grant, R. T. & Pearson, R. S. E. (1938). *Clin. Sci.* **3**, 119.

Lewis, T. & Grant, R. T. (1925). *Heart*, **12**, 73.

McMichael, M. & Snyder, C. H. (1941). *Amer. Nat. Res. Coun. Med. Sci. Committee of Aviation Medicine*, Report No. 185.

J. Physiol. (1949) 108, 405–417

612.116.546.262.3.668.8

THE DETERMINATION OF BLOOD VOLUME BY THE CARBON MONOXIDE AND DYE (T-1824) METHODS IN RABBITS

By F. C. COURTICE and R. W. GUNTON

From the Laboratory of Physiology, University of Oxford

(*Received* 15 *June* 1948)

The carbon monoxide and dye methods of determining blood volume have been compared in man and found to give essentially the same value (Courtice & Gunton, 1949*a*). It was concluded that the CO method had certain advantages over the dye method, especially when repeated determinations were required. Similar advantages would be of value in experimental animals, so the two methods have also been further investigated in rabbits.

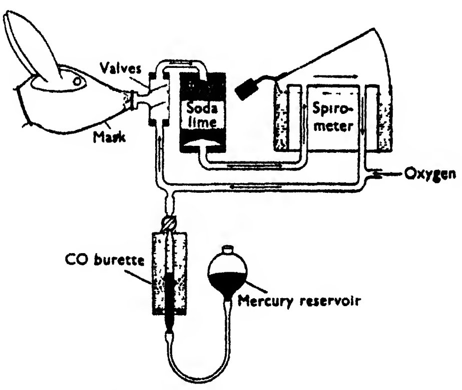


Fig. 1. Diagram of rebreathing circuit for blood volume estimation by CO method.

METHODS

The carbon monoxide method. The apparatus for administering CO to rabbits is illustrated in Fig. 1. It is a closed breathing circuit consisting of a 200 c.c. Krogh spirometer, glass soda-lime container, and respiratory valves made of thin rubber cemented to perforated rubber stoppers. Air is inspired from the spirometer and expired through the soda-lime back to the spirometer. This circuit appeared to cause no resistance to breathing. Unanaesthetized animals sat quietly at rest breathing normally, and easily increased the respiratory rate without apparent discomfort. The mask was made of thin black rubber and was so shaped that it fitted tightly to all parts of the animal's face. It was held in position by straps which extended back to the angle of the mandible

and the occiput, leaving an aperture for the ears and eyes. The anterior end of the mask was cemented tightly around a rubber stopper perforated by a glass tube which could be connected to the side arm of the valve unit. The inner surface of the mask was lined by a thin layer of compressible rubber which stopped about ½ in. short of the nostrils. With the mask in position the elasticity of the black rubber compressed this lining tightly against all irregularities in the animal's face and left a small free space surrounding the nostrils. Masks of this design were made in two sizes. They provided a suitable fit for adult rabbits because the size and shape of the head of these animals is fairly uniform. The possibility of a significant leak was excluded by several tests. The mask was placed in position and the tube leading from the mask to the valve unit was tightly clamped. The animals soon became asphyxiated. In another test the animal was anaesthetized and its head, with the mask in place, was immersed in a water-bath, with a length of rubber tubing connecting the interior of the mask to the breathing circuit. No water was aspirated into the mask. No bubbles of air appeared on expiration even when the possible sites of leakage were held 1 cm. below the surface, or when resistance to expiration was increased by placing a weight on the spirometer. In several experiments the blood volume was determined on a rabbit breathing through the mask, and later, breathing from an intra-tracheal cannula tied tightly in place. The agreement in results showed that no significant escape of CO had occurred during the course of the blood volume determination. The CO was admitted into the inspiratory side of the circuit from a gas burette immersed in a water jacket and connected to a mercury levelling bulb. The burette was calibrated in 0·05 c.c. divisions and could be read to 0·01 c.c. The CO was prepared, stored, analysed for purity and its volume corrected to S.T.P. as in the human experiments. For the rabbits a volume of approximately 3·0 c.c. was admitted for a blood-volume determination. This raised the CO content of the blood to 2·00 c.c./100 c.c. in an animal of average size. O_2 was admitted to the circuit at intervals to maintain the volume of the system constant.

Blood samples were taken from the marginal vein of the ear. Adequate blood flow was encouraged by warming the ear with an electric light for several minutes prior to the venipuncture; samples so obtained were essentially arterial in character. For the CO in blood analysis 1·5 c.c. samples were collected in small glass containers and transferred immediately to glass syringes for anaerobic storage. Powdered heparin was used as the anti-coagulant. Analyses were done in duplicate using the syringe capillary apparatus of Scholander & Roughton (1943).

In the experiments on unanaesthetized rabbits, the animals were placed in a box with the head outside. The box had an aperture in the front just large enough to fit the neck and prevent the head being withdrawn.

The dye method. For injection into rabbits the blue dye T-1824 (obtained from Messrs Eastman Kodak Co., U.S.A.) was dissolved in distilled water to make a concentration of 60 mg./100 c.c. This stock solution of dye was stored in a tightly stoppered flask. Its strength was checked frequently in a photoelectric colorimeter against a freshly prepared solution of the same concentration. The dye injection was made into the marginal vein of the rabbit's ear using a calibrated syringe. 2·0 c.c. of the stock solution were used for a blood-volume determination.

Blood samples were taken from the marginal vein of the opposite ear at intervals after the injection, with heparin as the anticoagulant. The blood samples were centrifuged for 15 min. at 2500 r.p.m. and the plasma was pipetted off: T-1824 concentration in plasma was determined in a photoelectric colorimeter from a calibration curve plotted from dye in rabbit plasma samples of known concentration. The calibration curve was checked several times throughout the course of the experiments. The detailed use of the colorimeter has already been described (Courtice & Gunton, 1949*a*). In the rabbit experiments it was not necessary to extract the T-1824 because rabbit plasma has a low pigment content and is usually clear.

The plasma dye concentration at 10, 20, and 30 min. after dye injection was plotted linearly in the form of a time-concentration curve. This curve was extrapolated to zero time to obtain the theoretical dye dilution at the moment of injection, assuming that mixing was complete in 10 min. and that the disappearance of dye from the circulation occurred at the same rate during the first 10 min. as during the succeeding 20 min. Plasma volume was calculated from dye dilution; total

blood volume from plasma volume and the venous haematocrit. The observed haematocrit was multiplied by the factor 0·96 to account for plasma trapped among the red cells (Gregersen & Schiro, 1938).

RESULTS

Uptake and disappearance of carbon monoxide. The rate of absorption of CO from the breathing circuit and its disappearance from the circulating blood during a 1 hr. rebreathing period were studied in seven anaesthetized and two unanaesthetized rabbits. The animals were anaesthetized with intravenous nembutal (1 grain/5 lb. of body weight) and the masks applied. O_2 was admitted to the spirometer and rebreathing commenced. A 'blank' sample of blood for the initial CO level was taken. The measured amount of CO was introduced into the circuit as rapidly as possible (10–15 sec.) and blood samples for CO analysis were taken at 3, 6, 9, 15, 30, 45 and 60 min. thereafter. The results are presented in Table 1. The initial blood samples in rabbits contained no CO except in

TABLE 1. Blood carbon monoxide content of rabbits breathing from closed circuit after the administration of carbon monoxide

Time (min.) ...	0	3	6	9	15	30	45	60
Anaesthetized	0	1·52	2·05	2·33	2·24	2·02	1·96	1·89
	0	1·63	1·95	2·22	2·23	2·13	2·00	1·97
	0	0·94	1·11	1·26	1·26	1·06	1·05	0·93
	0	1·52	2·38	2·94	3·05	2·87	2·70	2·51
	0	1·34	1·94	2·23	2·35	2·19	2·07	2·10
	0	1·99	2·62	2·75	2·98	2·86	2·82	2·62
	0	1·13	1·44	1·52	1·35	1·33	1·26	1·20
Mean	0	1·44	1·93	2·18	2·21	2·07	1·98	1·89
Unanaesthetized	0	—	1·81	1·74	1·80	1·77	1·68	1·62
	0	—	2·09	2·06	1·97	1·83	1·67	1·58
Mean	0	-	1·95	1·90	1·89	1·80	1·68	1·60

CO in c.c./100 c.c.

cases where the animal had been used recently for a blood-volume determination. In the anaesthetized animals there was a slow rise in the CO content of the blood through the 3 and 6 min. samples to a maximum at 9 or 15 min. From 15 min. to 1 hr. there was a slow fall which amounted to 14·3% of the maximum value, taking average figures from all experiments. It was shown in the human CO curves that the slow fall in CO concentration was not due to haemodilution. In rabbits the repeated removal of 1·5 c.c. samples of blood did result in a fall in haematocrit. In a corresponding series of experiments on anaesthetized rabbits this fall amounted to 3·3% of the original value, so that part of the disappearance effect observed was due to the dilution of the blood.

In the experiments on unanaesthetized rabbits appearing in Table 1, and in others done during the course of blood-volume determinations where the CO levels were followed for 15 min., the maximum blood-CO concentration was reached earlier, at 6 or 9 min., due to the increased pulmonary ventilation of the unanaesthetized animals. For the calculation of blood volume the CO

concentration at 9 min. was usually used, unless the animal breathed so little that the 15 min. value was higher. In most cases absorption of CO was complete at 6 min., so that use of the 9 min. value in the calculation allowed additional time for mixing, but did not include a significant part of the disappearance effect.

Carbon monoxide remaining in lung-spirometer system at equilibrium. The measurement of the amount of CO remaining unabsorbed at equilibrium is necessary for the accurate determination of blood volume by the CO method. This factor is of greater importance in rabbits than in humans because of the lower affinity of rabbit haemoglobin for CO. With a lower K value and higher oxygen tensions the residual CO at equilibrium given by the equation $pCO = \frac{HbCO \times pO_2}{HbO_2 \times K}$ would be expected to comprise a greater percentage of the CO admitted to the circuit. Experiments were conducted to determine the correction required. The procedure was the same as that for uptake and disappearance curves except that 80–100 c.c. samples of the air in the circuit were taken into gas sampling tubes at 10 and 30 min. after the administration of CO. The percentage of O_2 and CO in these samples was determined; O_2 by the Haldane gas analysis apparatus and CO by the technique described in the previous paper. The results are presented in Table 2. These showed some

TABLE 2. Carbon monoxide and oxygen content of air in breathing circuit at 10 and 30 min. after administration of carbon monoxide

Min.	CO (%)	O_2 (%)	Min.	CO (%)	O_2 (%)
10	0·0558	79·1	30	0·0420	90·2
30	0·0492	89·0	10	0·0467	62·1
10	0·0581	75·4	10	0·0269	53·3
10	0·0274	55·8	30	0·0306	77·6
30	0·0384	62·0			

variation in the determined CO percentage between individual rabbits and even in the same rabbit at 10 and 30 min. This CO percentage was modified by several factors. In several cases the O_2 tension was significantly higher at 30 min. In all cases the blood-CO content was lower at 30 min. than at 10 min. According to the equilibrium equation the CO tension would be expected to vary as the other values contributing to the equilibrium varied. In order to determine the correction factor to be applied to the blood-volume calculations, average values from these experiments were used. The average CO percentage was 0·0417%. The volume of the breathing circuit with the spirometer half-filled, the lungs, and the dead space of the mask and tubing, totalled 230 c.c. Thus the volume of CO unabsorbed equalled approximately 0·10 c.c. The usual volume of CO admitted to the circuit was 3·0 c.c. Therefore the unabsorbed CO comprised 3·3% of the CO admitted. A correction factor of 0·967 was applied to the blood-volume calculations.

This figure was obtained from average values. In experiments where the O_2 tension, HbCO saturation, and volume of CO admitted to the circuit were different from these average figures, this factor would not be strictly correct. However, the most extreme variations observed in the course of this work would not have affected the final figure for the blood volume by more than 1% of itself.

Blood volume by carbon monoxide method repeated. As a test of the accuracy of the CO method in rabbits the blood-volume estimation was repeated on the same animal within a short space of time. In three experiments the CO was administered and blood levels followed for a period of 30 min. Then while the animal continued to breathe from the closed circuit, a second dose of CO was admitted and the increase in blood-CO content estimated. In this way two determinations of blood volume were made within a period of 30 min. under identical conditions. The results appear in Table 3.

TABLE 3. Blood volume of rabbits in c.c. by carbon monoxide method repeated at 30 min.

	I			II		
Exp.	Total	Cell	Plasma	Total	Cell	Plasma
1	194	54	140	191	45	146
2	147	61	86	157	65	92
3	160	65	95	162	60	102

In another series of experiments the blood volume was determined by the CO method after an interval of 3–4 hr. The first determination was carried out in the usual way with rebreathing continued for 15 min. The animal was then removed from the apparatus and allowed to breathe room air for several hours. No food or water were given during this period. This interval of time allowed the animal to breathe off most of the CO which had been absorbed during the first determination. The blood-volume estimation was then done a second time. The amount of CO remaining in the animal's blood was measured as the 'blank' sample prior to administration of the second dose of CO. The haematocrit was repeated during the second determination in order to reveal any changes which might have occurred in the cell to plasma ratio during the interval. The results of three experiments are given in Table 4. Typical curves for the CO levels of the blood in individual experiments are shown in Fig. 2. These experiments showed that the CO method gave close agreement in values for the blood volume with repeated determinations. In several cases the haematocrit was lower during the second estimation and the total blood volume higher. This was probably a response to the removal of the blood samples. The red cell volume remained the same or several c.c. lower than the initial level. Lower values were anticipated because 1–3 c.c. of red cells were removed during the first determination.

TABLE 4. Blood volume of rabbits in c.c. by carbon monoxide method repeated at 3–4 hr.

	I			II		
Exp.	Total	Cell	Plasma	Total	Cell	Plasma
1	141	41	100	142	39	103
2	136	51	85	148	51	97
3	168	62	106	174	58	116

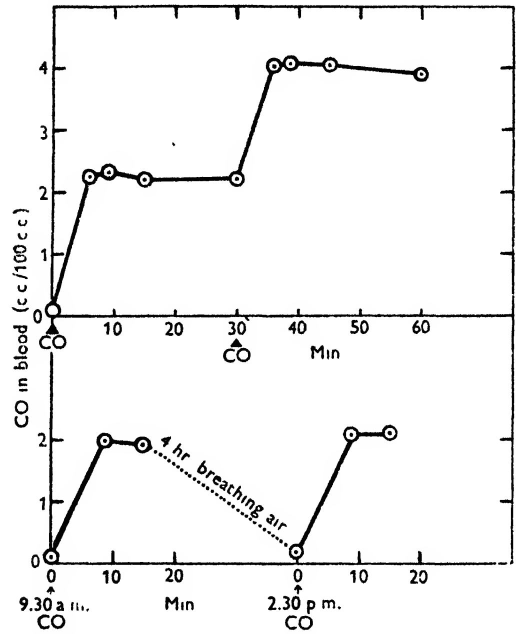


Fig. 2. CO curves in repeated estimations of blood volume in single experiments. Upper curve: estimation repeated immediately after the first. Lower curve: estimation repeated 4 hr. after the first.

Disappearance curve of T-1824 *after single and repeat dye injection.* It has already been pointed out that because T-1824 disappears slowly from the circulation, the theoretical dye concentration at the moment of injection is obtained by extrapolation of the disappearance curve back to 0 min. These curves have been studied in man and dogs by various investigators. They commonly show an initial rapid fall in dye concentration occupying the first 10 min. which is attributed to mixing, followed by a linear disappearance 'slope' up to 1 hr. Recently Cruickshank & Whitfield (1945) have questioned the validity of this interpretation. They have pointed out that T-1824 is taken up by the reticulo-endothelial system and believe the early rapid fall in dye concentration is due to 'gobbling' of dye by this tissue. To support this claim they have 'blocked' the reticulo-endothelial system by an injection of T-1824 or indian ink, and have found in the cat that a second injection of dye does not

show the early rapid fall. They have suggested that the blood volume estimated from a single dye injection gives falsely high values. In order to study this phenomenon in rabbits, disappearance curves of T-1824 were followed in anaesthetized animals for 60 min.; a repeat dye injection was made, and the dye concentration followed for a further period. Time-concentration curves were plotted; plasma volumes calculated from the original and repeat dye injections were compared. 2·0 c.c. of dye solution were injected in the usual manner; 1·5 c.c. blood samples were taken at 0, 2, 4, 6, 9, 15, 30, 45, 60 min.; the dye injection was repeated at 60 min.; further samples were taken at 62, 64, 66, 69, 75 and 90 min.

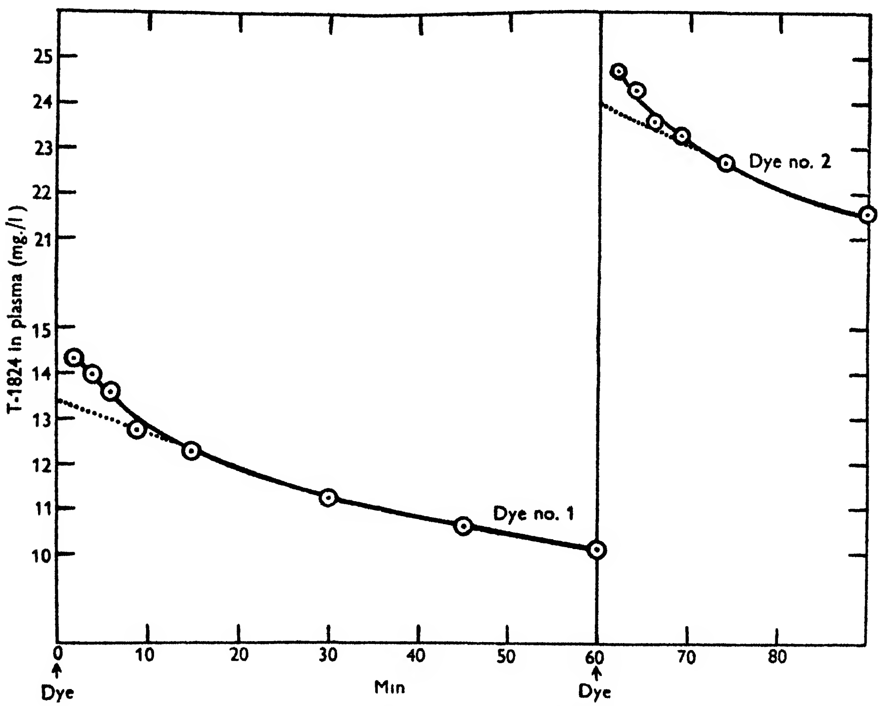


Fig. 3. Dye concentration in plasma after two injections of dye.

In each of the five experiments performed the dye disappearance curve after the repeat dye injection showed the same initial rapid fall during the first 10 min. followed by a linear disappearance slope as occurred after the first dye injection. The results of one experiment are shown in Fig. 3. The method of extrapolation of the curve to zero time from 10 min. is illustrated. The total, red cell, and plasma volumes in each of the five experiments calculated by this extrapolation procedure are given in Table 5. In each of the five experiments the blood volume calculated from the second dye injection was several c.c. lower than that calculated from the first. On the average it was 9·0 c.c. lower.

This observation will bear further scrutiny. During the first 60 min. a significant amount of blood was removed for the dye and haematocrit determinations—approximately 14·0 c.c. If no replacement of fluid had occurred from the extra-vascular spaces the blood volume should have been estimated 14·0 c.c. lower by the second dye injection and the haematocrit should have remained constant. The figures show that at the end of the hour the haematocrit was 4% lower than the initial value, indicating that fluid had been added to the circulation. Using average figures, this would have been 5·5 c.c. That is, 14·0 c.c. of blood were removed and 5·5 c.c. of fluid replaced to the moment of the second blood-volume determination—a net reduction of 8·5 c.c. The average reduction measured by the second dye injection was 9·0 c.c. Further confirmation of the accuracy of the repeat determination by the dye method was obtained by examining red cell volume figures. In each experiment, and on the average, a reduction in red cell volume of 5·0 c.c. would be expected.

TABLE 5. Blood volume of rabbits by dye method repeated at 60 min.

	I				II			
Exp.	Haematocrit at 0 min.	Plasma	Cell	Total	Haematocrit at 60 min.	Plasma	Cell	Total
1	27·9	103	39	142	26·3	104	36	140
2	39·4	83	55	138	39·8	77	52	129
3	33·9	85	43	128	31·8	80	37	117
4	33·2	88	44	132	32·2	85	40	125
5	34·2	102	53	155	31·5	95	44	139
Mean	33·7	92	47	139	32·3	88	42	130

Volumes in c.c.

These results show that in the rabbit the blood volume determination by a repeat dye injection is valid. Failure to account for the amount of blood removed during the first estimation and the effects of haemodilution might cause erroneous interpretation of results in such small animals as the cat and rabbit.

Simultaneous determination of blood volume by carbon monoxide and T-1824. Because CO is combined with haemoglobin, the CO method measures red cell volume directly. T-1824 is mixed with circulating plasma and does not enter the red cells; the dye method therefore measures plasma volume directly. Total blood volume in each case is calculated on the assumption that the venous haematocrit truly represents the ratio of the body's total red cell volume to plasma volume. If this assumption is correct simultaneous determination of blood volume by CO and dye methods should give equivalent values.

In fifteen experiments the blood volume of unanaesthetized rabbits was determined simultaneously by CO and dye methods. With the animal breathing from the closed circuit, the CO was admitted and the dye solution injected into an ear vein at the same moment. The results are presented in Table 6.

In these fifteen experiments, eleven of the comparisons showed agreement between the two methods within 5%, and in only one case was the discrepancy greater than 10%. The average ratio of CO-blood volume to dye-blood volume was 1·02. In terms of blood volume per unit of body weight the average results for these rabbits by the CO method were: cell volume 18·0 c.c./kg.; plasma volume 39·8 c.c./kg.; total blood volume 57·7 c.c./kg. Similar values were obtained in twenty other animals where either the CO method or dye method was used independently.

TABLE 6. Blood volume of rabbits by carbon monoxide and T-1824 methods simultaneously

Rabbit	Wt. (kg.)	Haematocrit	CO Plasma	CO Cell	CO Total	T-1824 Plasma	T-1824 Cell	T-1824 Total	Total vol. CO / Total vol. dye
1	2·65	22·3	112	32	144	112	32	144	1·00
2	2·30	20·2	111	28	139	100	25	125	1·11
3	2·40	21·5	111	30	141	115	32	147	0·96
4	2·55	38·3	86	54	140	91	56	147	0·95
5	2·80	34·7	102	54	156	101	54	155	1·01
6	3·10	34·4	107	56	163	98	51	149	1·09
7	2·10	37·2	84	49	133	81	48	129	1·03
8	2·65	32·5	90	43	133	85	41	126	1·06
9	2·60	30·1	100	43	143	93	40	133	1·08
10	2·60	26·6	138	50	188	147	53	200	0·94
11	2·40	37·3	83	49	132	79	47	126	1·05
12	2·50	39·0	86	55	141	88	56	144	0·98
13	2·55	35·7	88	49	137	92	51	143	0·96
14	2·10	30·5	96	42	138	91	40	131	1·05
15	2·50	30·3	121	53	174	123	53	176	0·99
Mean	—	—	—	—	—	—	—	—	1·02

Plasma, cell and total volume in c.c.

Test of accuracy of the carbon monoxide method by the removal and addition of known volumes of red cells. In the preceding experiments the CO and dye methods were studied independently and compared. It was considered possible that although they gave results which agreed closely, there could have been systematic errors in each which caused the blood volume by both methods to be estimated higher or lower than the true value. It was felt that a more direct test of the accuracy of either method would be to measure the reduction or increase in blood volume resulting from the removal or addition of known amounts of blood. For this purpose the CO method was used in unanaesthetized rabbits. The blood volume was determined in the usual way at about 10.00 a.m. Approximately 40·0 c.c. of blood were removed by bleeding from the marginal vein of the ear. This blood was collected in a small flask; coagulation was prevented by dry heparin. The volume removed was measured in a graduated cylinder; a small sample was saved for the haematocrit determination and the remainder was stored overnight in a refrigerator. The rabbit was kept in the laboratory without food or water, and a second blood-volume determination was done 4 hr. later. Unanaesthetized rabbits restore their circulating fluid volume very rapidly after haemorrhage (Courtice & Gunton, 1949*b*). It was

possible therefore to compare only changes in red cell volume. Haematocrits were done in duplicate on the mixed sample of blood which had been removed and on the circulating blood during each blood volume determination.

In another series of experiments, blood which had been removed from one rabbit was transfused slowly into another rabbit. Red cell volume was estimated before and after addition of this known amount of red cells to the circulation. The results of these experiments are given in Table 7. They provided satisfactory evidence that the CO method of determining blood volume in rabbits described in this work was accurate. The estimated reduction or increase in red cell volume was within 1·0–1·5 c.c. of the volume of cells known to have been removed from or added to the circulation.

TABLE 7. Addition and removal of known volumes of red cells

Exp.		Original red cell volume	Final red cell volume	Estimated reduction or increase in red cell volume	Volume of red cells actually removed or added
Bleeding	1	56·6	42·6	14·0	14·1
	2	56·7	41·9	14·8	13·6
	3	58·2	43·2	15·0	15·4
	4	44·2	30·5	13·7	14·8
Transfusion	1	33·8	46·3	12·5	11·6
	2	48·3	61·9	13·6	12·3
	3	45·2	58·8	13·6	13·0

Volumes in c.c.

The effect of diet on the blood volume of rabbits. Cameron & Courtice (1946) studied the effect of phosgene on the production of lung oedema, plasma volume, and blood concentration in rabbits. They found that in rabbits maintained on a normal diet the average excess oedema fluid in the lungs 6 hr. after exposure to the irritant gas was 31 c.c. Although this fluid had been derived from the circulating plasma, they found that the plasma volume was not decreased and there had been no haemoconcentration in the 6 hr. period. They concluded that the rabbit normally has a readily available store of fluid with which lost plasma can be rapidly replaced. These observations are corroborated by the work on haemorrhage reported in another paper (Courtice & Gunton, 1949*b*) in which it was shown that rabbits can replace 30 c.c. of plasma in 2 hr. Cameron & Courtice studied these same changes in response to phosgene on rabbits whose fluid intake had been restricted for several days prior to the experiment. They found that lung oedema developed as in the other cases, but it was accompanied by haemoconcentration, indicating failure to replace plasma rapidly because of depletion of the animal's fluid stores. It was felt that it would be of interest to study the effect of variation in the water content of the food on the plasma volume of rabbits not subjected to any experimental procedure. Six animals were used in these experiments, two groups of three. One group was fed on dry oats and bran with water *ad lib.*

The second group was given the same diet with the addition of an excess of cabbage. After 3 days on these diets the blood volume of all six rabbits was determined by the CO method. Then, excess of cabbage was given to the first group and withdrawn from the second group. After a further 3 days on these diets the blood volume of all six rabbits was again determined. Although water was freely available the animals drank very little. Because the water content of cabbage is very high and rabbits eat this green food voraciously, the fluid intake on the cabbage diet was increased considerably.

TABLE 8. Effect of diet on the blood volume of rabbits

		Normal diet				Cabbage diet			
Rabbit	Wt. (kg.)	Haematocrit	Cell	Plasma	Total	Haematocrit	Cell	Plasma	Total
1	2·40	34·3	44	83	127	30·2	44	102	146
2	2·50	33·2	39	77	116	28·7	39	96	135
3	2·90	29·9	61	143	204	29·5	60	143	203
4	2·80	29·0	45	110	155	28·9	50	124	174
5	2·55	32·4	49	101	150	28·5	52	129	181
6	2·70	31·6	45	96	141	30·0	45	104	149
Mean	2·65	31·7	47	102	149	29·3	48	117	165

Volumes in c.c.

The results of the experiments are presented in Table 8. There was no significant change in the red cell volume between the dry and the cabbage diet. However, in five of the six animals, there was an increase in the plasma and total blood volume. The increase averaged 15 c.c., that is 10% of the original blood volume, or 15% of the original plasma volume. A corresponding fall in the haematocrit was recorded. Undoubtedly the increase of plasma volume was paralleled by an increase in the fluid stores of the body so that the compensatory mechanisms for restoration of circulating fluid in haemorrhage and shock are greater on a wet diet. It is not possible to say whether this effect would be present in other species such as the cat, dog and human without further study. Rabbits appear to be capable of wide variations in fluid adjustment.

DISCUSSION

In estimating the blood volume of a small animal like the rabbit, the blood samples taken must be kept as small as possible; otherwise a significant percentage of the blood volume will be withdrawn in this way. In the rabbit where haemodilution and overcompensation occur rapidly, this factor may introduce errors if several samples are taken. Consideration of the fall in concentration of CO in the blood or of dye in the plasma must therefore involve this dilution effect. The fall in the concentration of CO in the blood of rabbits during a period of an hour on the rebreathing circuit is partly due to the removal of the blood samples and reabsorption of tissue fluid to dilute the blood. This, however, is not the only cause of the fall in CO concentration. The

disappearance of CO from the circulating blood observed in this work in rabbits has also been reported in man (Roughton & Root, 1945; Courtice & Gunton, 1949*a*). Roughton & Root have shown that this is due to reversible combination of CO with some haemoglobin-like substance outside the circulation. It is important in the determination of blood volume by the CO method only if it causes the uptake of a significant amount of CO in the first few minutes required for complete absorption of the gas from the closed breathing circuit. It was concluded (Courtice & Gunton, 1949*a*) that the combination of CO with myoglobin was a slow process and was represented by the gradual fall in CO content of the blood over the 1 hr. period. This would mean that calculation of the blood volume from the CO concentration 10 or 15 min. after the commencement of rebreathing would not include a significant part of the disappearance effect. If a large fraction of the CO was taken up by myoglobin in this early period, the blood volume by the CO method would be calculated too high. The very close agreement between the estimated change in red cell volume and the volume of cells actually removed from, or added to, the circulation in the rabbit experiments provided strong evidence that this source of error was not significant.

In using the CO method the rate of uptake and disappearance of CO should always first be determined for the circuit used and the circumstances under which the experiment is done. For example, the pulmonary ventilation will determine the rapidity of uptake of CO, and this will probably be much greater in unanaesthetized than in anaesthetized animals.

The results of the simultaneous determinations of blood volume by CO and T-1824 are in accord with those previously reported for humans (Courtice & Gunton, 1949*a*) and with those of Root, Roughton & Gregersen (1946). They indicate that there is no significant difference between central venous and 'body' haematocrit.

Most recent work on blood volume has been done using the T-1824 method. Emphasis has been placed here on the CO method. Use of a standard closed-circuit breathing system and the syringe-capillary method of CO analysis has made the procedure simple and accurate. The accuracy has been tested by comparison with the dye method, by repeat determinations and by the addition and removal of known amounts of blood. It has been found that the CO method offers certain advantages over the dye method. Only two small blood samples are required, haemolysis in these is of no importance and the method can be repeated several times in one day with the same degree of accuracy. These considerations recommend the CO method for use in clinical or laboratory investigations, where it is desired to perform repeated determinations of the blood volume in a short period of time.

SUMMARY

1. The carbon monoxide method of determining blood volume has been further investigated in rabbits and compared with the dye (T-1824) method.

2. No significant difference was found between the values obtained by both methods.

3. The accuracy of the carbon monoxide method was tested by estimating the blood volume before and after the removal of a known volume of blood.

4. Variation of the fluid content of the diet was shown to affect the plasma volume significantly.

We are indebted to Mr P. J. Phipps for his technical assistance.

REFERENCES

Cameron, G. R. & Courtice, F. C. (1946). *J. Physiol.* **105**, 175.

Courtice, F. C. & Gunton, R. W. (1949*a*). *J. Physiol.* **108**, 142.

Courtice, F. C. & Gunton, R. W. (1949*b*). *J. Physiol.* **108**, 418.

Cruickshank, E. W. H. & Whitfield, I. C. (1945). *J. Physiol.* **104**, 52.

Gregersen, M. I. & Schiro, H. (1938). *Amer. J. Physiol.* **121**, 284.

Root, W. S., Roughton, F. J. W. & Gregersen, M. I. (1946). *Amer. J. Physiol.* **146**, 739.

Roughton, F. J. W. & Root, W. S. (1945). *Amer. J. Physiol.* **145**, 239.

Scholander, P. F. & Roughton, F. J. W. (1943). *J. biol. Chem.* **148**, 551.

J. Physiol. (1949) 108, 418–426 615.782.54:612.116.2

EFFECT OF NEMBUTAL ANAESTHESIA ON RESTORATION OF PLASMA VOLUME AFTER HAEMORRHAGE IN DOGS, CATS AND RABBITS

By F. C. COURTICE and R. W. GUNTON

From the Laboratory of Physiology, University of Oxford

(*Received* 15 *June* 1948)

During an investigation into the effects of haemorrhage on lymph flow and other phenomena in dogs anaesthetized with nembutal, it was observed that after removing about 20% of the animal's blood volume, no haemodilution occurred during the ensuing 4–6 hr. On the other hand, in unanaesthetized dogs, a similar haemorrhage was followed in this time by definite haemodilution. While studying the carbon monoxide and T-1824 disappearance curves in the rabbit, Courtice & Gunton (1949*b*) also noted that nembutal influenced the rate of haemodilution after the removal of small blood samples at intervals during the course of an hour. In the series of nembutalized animals a fall in the haematocrit of approximately 5% occurred, whereas in unanaesthetized rabbits a decrease up to 20% of the original was noted, although similar amounts of blood at the same time intervals were removed.

Since nembutal is commonly used as an anaesthetic for experimental animals, it was decided to investigate its effects on the restoration of the blood volume after haemorrhage in dogs, cats and rabbits.

METHODS

The reabsorption of fluid into the circulation after haemorrhage has been studied by determinations of the haemoglobin percentage, haematocrit, plasma protein concentration and blood volume before and after removal of a known amount of blood. These experiments were performed on unanaesthetized dogs and rabbits, and on dogs, cats and rabbits under nembutal anaesthesia. Anaesthesia was maintained for 4–6 hr. after which the cats were killed, but the dogs and rabbits were allowed to recover and determinations repeated after 24 hr.

Abbot's veterinary nembutal (pentobarbital sodium) containing 1 grain/c.c. was used throughout. It was administered intraperitoneally in dogs and cats, and intravenously in rabbits, in doses of 1 c.c./5 lb. or sufficient to anaesthetize the animal. Repeated smaller doses were given when necessary to keep the animals anaesthetized for the required time.

Dogs, both unanaesthetized and anaesthetized, were bled from the jugular vein by venepuncture and slight suction by negative pressure. The volume removed was 16 c.c./kg. which, according to the figures of Courtice (1943), represents about 20% of the blood volume. The blood samples were also taken from the jugular vein by venepuncture. Cats were bled from a cannula in the femoral artery, and blood samples were taken in the same way. Rabbits were bled from the marginal vein

of the ear. In all experiments food and water were withheld from the unanaesthetized animals for 4–6 hr. after bleeding, the time comparable to the period of anaesthesia in the anaesthetized animals.

Analytical methods. The haemoglobin percentage was determined by the Haldane haemoglobinometer, and the plasma proteins by micro-Kjeldahl digestion and nesslerization. The blood volume in dogs was measured by the dye (T-1824) method described by Courtice (1943) and in rabbits by the CO method described by Courtice & Gunton (1949 *b*). The blood volume of anaesthetized cats was also determined by the CO method, using the apparatus for rabbits. Instead of a mask, a tracheal cannula was inserted and connected to the rebreathing circuit. In each of the four cats used, the CO concentration of the blood was estimated at 10, 20 and 30 min. after administration of CO to the closed circuit. The results of these experiments are given in Table 1. In the cat, as in man and rabbit, there is on the average a gradual fall in the CO content of the blood after from 10 to 30 min. The blood volume has been calculated from the 10 min. figure.

TABLE 1. The carbon monoxide content of the blood in anaesthetized cats before and at intervals after the administration of carbon monoxide in the closed rebreathing circuit. Carbon monoxide in c.c. at S.T.P./100 c.c.

Time (min.) ...	0	10	20	30
1	0·09	1·83	1·77	1·68
2	0·06	2·24	2·04	1·96
3	0·18	2·02	2·06	2·07
4	0·12	1·81	1·76	1·76
Mean	0·11	1·98	1·91	1·87

RESULTS

Effect of nembutal anaesthesia on blood of dogs and rabbits. It has been shown by previous investigators (Bollman, Svirbley & Mann, 1938; Hahn, Bale & Bonner, 1943; Jarcho, 1943) that barbiturate anaesthesia causes a decrease in red cell volume, an increase in plasma volume, a fall in plasma protein concentration and an increase in the size of the spleen. These changes have been confirmed in dogs as shown by average results in Table 2. The effect on rabbits is much less pronounced, the degree of dilution being less than that observed in the dog.

TABLE 2. The effect of nembutal anaesthesia on the blood of normal dogs and rabbits

	Before nembutal	Hours after nembutal ½	1	2	4
	(*a*) Mean of four dogs				
Haemoglobin (%)	122	109	105	106	109
Haematocrit	42·5	38·9	—	—	37·3
Plasma proteins (g./100 c.c.)	6·2	6·0	5·8	5·7	5·9
	(*b*) Mean of five rabbits				
Haemoglobin (%)	74	72	73	74	74
Plasma proteins (g./100 c.c.)	5·6	4·9	5·3	5·3	5·5

(*c*) Mean of six dogs

	Before nembutal	½ hr. after nembutal
Total blood vol. (c.c.)	1463	1530
Cell vol. (c.c.)	638	606
Plasma vol. (c.c.)	825	924

The difference in behaviour of these two animals may in part be due to the relative size of the spleen. The dog is known to have a relatively large spleen, so that the deposition of red cells in the spleen after nembutal is probably greater than in the rabbit. That the spleen plays a large part in the effect of nembutal on red cell volume can also be seen in the reaction of splenectomized dogs to nembutal. In these animals no significant fall in haemoglobin percentage occurs after nembutal (Table 2). The action of nembutal on the blood is rapid and, once haemodilution occurs, the haemoglobin percentage remains fairly constant at the lower level for at least 4 hr. with the animal anaesthetized all the time.

Haemorrhage experiments in the dog. Dogs were bled 16 c.c./kg. fairly rapidly, usually 5–15 min. In Table 3 are shown the rates of haemodilution in unanaesthetized and anaesthetized animals. In unanaesthetized dogs the haemoglobin percentage falls to a minimum in 6–24 hr. on the average. In nembutalized dogs, on the other hand, there is no fall, but in individual experiments sometimes a rise in the haemoglobin percentage and haematocrit during the 4–6 hr. of anaesthesia. Next day, when the animal has recovered from the anaesthetic, haemodilution has been observed as expected.

TABLE 3. Effect of bleeding 16 c.c./kg. in normal dogs

(*a*) Unanaesthetized

	Group 1 (mean of 4 dogs)		Group 2 (mean of 6 dogs)		
	Hb (%)	Plasma proteins (g./100 c.c.)	Hb (%)	Haematocrit	Plasma proteins (g./100 c.c.)
Before bleeding	102	6·0	109	39·6	6·0
1 hr. after bleeding	88	5·5	96	—	5·9
2 ,, ,,	87	5·5	96	—	5·7
4 ,, ,,	85	5·3	95	34·4	5·4
6 ,, ,,	82	5·2			
24 ,, ,,	81	5·6			
48 ,, ,,	84	6·2			

(*b*) Anaesthetized

	Group 1 (mean of 7 dogs)		Group 2 (mean of 6 dogs)		
	Hb (%)	Plasma proteins (g./100 c.c.)	Hb (%)	Haematocrit	Plasma proteins (g./100 c.c.)
Before nembutal	—	—	116	41·6	5·7
After nembutal	96	5·4	104	37·3	5·5
1 hr. after bleeding	98	5·0	106	—	5·2
2 ,, ,,	99	5·0	106	—	—
4 ,, ,,	100	4·8	107	37·2	4·9
6 ,, ,,	100	4·8			
24 ,, ,,	88	4·9			
48 ,, ,,	88	5·1			

In two groups of dogs the blood volume has been measured before and 4 hr. after haemorrhage. Table 4 shows that whereas about half the plasma removed

has been restored in this time in unanaesthetized dogs, no restoration has been made in the anaesthetized animals. It will also be observed that in neither group is there an increase in red cell volume, which indicates that with this degree of haemorrhage there is no significant contraction of the spleen. The failure of the haemoglobin percentage to fall after haemorrhage in the anaesthetized dogs is, therefore, not due to a contraction of the spleen. This

TABLE 4. The effect of haemorrhage on the blood volume of unanaesthetized and anaesthetized dogs

	B.V. before bleeding (c.c.)	Blood removed (c.c.)	Theoretical B.V. immed. after (c.c.)	Actual B.V. 4 hr. after (c.c.)
	(*a*) Mean of four unanaesthetized			
Total	1204	238	966	1013
Plasma	724	143	581	648
Cells	480	95	385	365
	(*b*) Mean of four anaesthetized			
Total	1586	351	1235	1224
Plasma	974	215	759	761
Cells	611	136	475	463

can also be shown by observing the effect of haemorrhage in previously splenectomized dogs under nembutal. Here, there is no haemodilution after nembutal, and also no significant restoration of plasma volume after haemorrhage while the animal is anaesthetized. When the anaesthetic has worn off, haemodilution occurs as shown in Table 5.

TABLE 5. The effect of bleeding 16 c.c./kg. in dogs under nembutal anaesthesia for 6 hr. and splenectomized 2–3 weeks previously (mean of 4)

	Hb (%)	Plasma proteins (g./100 c.c.)
Before nembutal	96	5·6
After nembutal	99	5·4
1 hr. after bleeding	95	5·0
2 ,, ,,	94	4·8
4 ,, ,,	95	5·0
6 ,, ,,	95	4·8
24 ,, ,,	77	5·1
48 ,, ,,	79	5·5

Haemorrhage experiments in the cat. Experiments have been carried out only in anaesthetized cats. The blood volume of a cat under nembutal anaesthesia was determined by the CO method, a measured volume of blood removed and the blood volume again determined 2 hr. after haemorrhage. The haemoglobin percentage and haematocrit were further determined at 4 hr. The mean results of four experiments are given in Table 6. It can be seen that no restoration of the plasma volume has been made during this time and no increase in red cell volume has occurred, indicating that the spleen has not added to the circulating red cells. Thus the anaesthetized cat behaves in a manner similar to the dog after haemorrhage.

TABLE 6. The effect of haemorrhage in cats under nembutal anaesthesia (mean of 4)

	Before haemorrhage	Hours after haemorrhage 1	2	4
Haemoglobin (%)	76	77	75	73
Haematocrit	32·6	33·3	32·2	31·6
Total B.V. (c.c.)	187	—	135	—
Plasma vol. (c.c.)	127	—	92	—
Cell vol. (c.c.)	60	—	43	—

Actual blood removed: Total 52 c.c.
Plasma 35 c.c.
Cells 17 c.c.

Haemorrhage experiments in the rabbit. In a preliminary series of experiments, the effects of bleeding approximately 14 c.c./kg. on the haemoglobin percentage and plasma protein concentration were determined in three groups of rabbits: (*a*) unanaesthetized, (*b*) anaesthetized, and (*c*) splenectomized 2–3 weeks earlier and anaesthetized. The average results are given in Table 7. In the anaesthetized groups the animals were kept under nembutal for 4 hr. and then allowed to recover. These results showed a rapid haemodilution in the unanaesthetized group and a slower haemodilution in the anaesthetized groups. Splenectomy appeared to have little or no effect.

TABLE 7. Effect of bleeding 14 c.c./kg. in rabbits (mean of 5 in each group)

Unanaesthetized	Hb (%)	Plasma proteins (g./100 c.c.)
Before bleeding	82	5·5
1 hr. after bleeding	71	4·6
2 ,, ,,	66	4·2
4 ,, ,,	66	3·9
24 ,, ,,	62	5·6

Anaesthetized	Normal Hb (%)	Normal Plasma proteins (g./100 c.c.)	Splenectomized Hb (%)	Splenectomized Plasma proteins (g./100 c.c.)
Before nembutal	78	4·8	76	5·2
After nembutal	75	4·3	74	4·6
1 hr. after bleeding	70	4·1	69	4·2
2 ,, ,,	70	3·9	67	3·9
4 ,, ,,	71	4·0	66	3·8
24 ,, ,,	61	5·1	59	5·2

In a second series of experiments the blood volume was determined by the CO method. Ten rabbits were used, five unanaesthetized and five anaesthetized with nembutal. Anaesthesia was maintained for 4 hr. and the animals then allowed to recover. As soon as the blood volume was estimated, the animals were bled from an ear vein. This usually required 10–20 min. and an amount of blood equal to approximately 30% of the blood volume was removed in each case. This blood was collected in a flask containing dry powdered heparin, its volume measured and haematocrit determined. Taking the mid-point of the bleeding period as zero time, small samples for haemoglobin and haematocrit determinations were withdrawn at 1, 2, 6 and 24 hr. The amount of blood taken for the preliminary blood volume estimation and for the 1 and 2 hr. haematocrit were included in the calculation for total volume of blood removed. The blood volume determination was repeated at 2 and 24 hr. in each of the ten animals.

Average values for the blood volume in the two groups before and 2 and 24 hr. after haemorrhage are given in Table 8. These experiments show how rapidly the unanaesthetized rabbit restores its plasma volume after haemorrhage. The average plasma volume of five rabbits was 104 c.c. before haemorrhage, and 2 hr. after removing 30 c.c. of plasma, the volume was 118 c.c. and

TABLE 8. The effect of haemorrhage on blood volume, c.c., in unanaesthetized and anaesthetized rabbits

	B.V. before	Volume removed	Theoretical B.V. after	Actual B.V. at 2 hr.	Actual B.V. at 24 hr.
		(a) Unanaesthetized (mean of 5)			
Total	153	43	110	153	157
Plasma	104	30	74	118	124
Cells	49	13	36	35	33
		(b) Anaesthetized (mean of 5)			
Total	136	40	96	126	159
Plasma	87	27	60	90	124
Cells	49	13	36	36	35

increased to 124 c.c. at 24 hr. The accuracy of the blood volume determinations can be seen from the cell volumes, the actual determined cell volume corresponding very closely to the theoretical cell volume after haemorrhage.

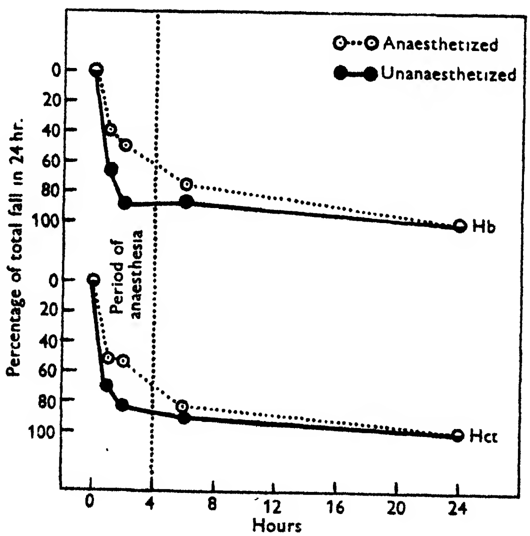


Fig. 1. The fall in haemoglobin percentage and haematocrit after haemorrhage in anaesthetized and unanaesthetized rabbits. The fall is expressed as a percentage of the decrease at 24 hr. Mean of five experiments in each group.

In the anaesthetized group the initial plasma volume was 87 c.c., and 2 hr. after removing 27 c.c. plasma the plasma volume was 90 c.c. and this increased

to 124 c.c. at 24 hr. Thus, although haemodilution occurred in the anaesthetized rabbit after haemorrhage, it was slowed down and increased when the animal recovered from the anaesthetic.

The difference between the two groups can also be well seen in the changes in haematocrit and haemoglobin percentage. In Fig. 1 are plotted the percentage fall in haematocrit and haemoglobin percentage, taking the fall from zero to 24 hr. as 100%.

DISCUSSION

These experiments on haemorrhage suggest several points of physiological interest. The first of these concerns the changes in circulating red cell volume following bleeding. It has been generally recognized that while a large proportion of the body's total red cells are in active circulation, there are reserve depots of red cells in such tissues as the spleen, liver sinusoids and bone marrow where the blood flow is sluggish or even stagnant (Wiggers, 1944). Barcroft and his associates (Barcroft, Harris, Orahovats & Weiss, 1925) focused attention on the spleen as one of the most important blood reservoirs which contracted during muscular exercise and haemorrhage. Other workers have observed splenic contraction and a rise in haematocrit following injection of adrenaline (Izquierdo & Cannon, 1928). There can be little doubt that these phenomena actually occur, but the fact that the spleen contributes a significant volume of red cells to the circulation has largely been inferred. Direct measurement of the red cell volume would provide the only satisfactory proof.

The results presented here do not demonstrate any increase in circulating red cell volume following non-fatal haemorrhage in the rabbit, cat and dog. The observations on rabbits and cats were made by the CO method, those on the dog by the dye method. The red cell volume after haemorrhage was always approximately equal to the original red cell volume less the volume of cells removed during the haemorrhage. It would appear, therefore, that in the degree of haemorrhage used here the reserve depots of red cells play an insignificant part in restoring blood volume. These observations in animals are supported by the work of Ebert, Stead & Gibson (1941) in man, where the red cell volume 2 hr. after bleeding approximately 1000 c.c. was equal to the original value less the amount of cells removed.

Concerning muscular exercise, Courtice & Gunton (1949*a*) showed that moderate work did not affect the red cell volume. Nylin (1947), using radioactively tagged red cells, has reported no increase in circulating red cell volume in man during heavy muscular exercise.

Another point of interest is the rate of replacement of fluid to the circulation following haemorrhage in unanaesthetized and anaesthetized animals. Since there is no replacement of cells, the plasma fraction of the blood is responsible for the early return of the circulating volume to normal. In Fig. 2 the changes in plasma volume in the cat and rabbit at 2 hr., and in the dog at 4 hr., after

haemorrhage are presented schematically. There is a very striking difference between the species. The rabbit replaces in 2 hr. more plasma than was removed in both anaesthetized and unanaesthetized groups, although this overcompensation is greater in the unanaesthetized animals. The unanaesthetized dogs have replaced part of the plasma removed, but the nembutalized cats and dogs have restored no fluid. It is probable that human subjects resemble cats and dogs in the rate of replacement of circulating fluid volume. Blood loss in hospital patients under barbiturate anaesthesia would, therefore, not be compensated for until recovery from the anaesthetic. This phenomenon is probably of practical importance in the choice of anaesthesia for surgical procedures in patients suffering from traumatic shock.

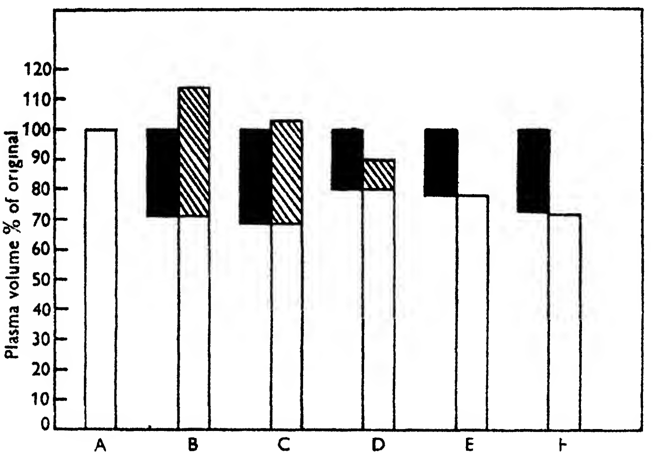


Fig. 2. The changes in plasma volume in rabbits and cats 2 hr. after haemorrhage, and in dogs 4 hr. after haemorrhage. In each case the original plasma volume is taken as 100. The black areas represent the volume of plasma removed in haemorrhage and the shaded areas the amount replaced in the stated times. A, original plasma volume; B, rabbits, unanaesthetized (mean of 5); C, rabbits, anaesthetized (mean of 5); D, dogs, unanaesthetized (mean of 4); E, dogs, anaesthetized (mean of 4); F, cats, anaesthetized (mean of 4).

The mechanism by which nembutal inhibits haemodilution is not clearly understood. Restoration of fluid to the circulation following haemorrhage in unanaesthetized animals is believed to be due to a fall in capillary pressure, with the result that fluid passes into the blood stream from the tissues. The fall in capillary pressure is brought about by peripheral arteriolar constriction, a reaction which maintains arterial blood pressure as blood is lost during haemorrhage. It is known that barbiturates cause arteriolar relaxation through depression of sympathetic tone. This would result in a raised capillary pressure. The osmotic effect of the plasma proteins would then be counterbalanced by the increased hydrodynamic pressure in the capillaries, thus slowing or stopping haemodilution.

The very rapid haemodilution in the rabbit, compared with the dog, is probably due to the more readily available tissue fluid stores of the rabbit. Cameron & Courtice (1946) have shown that in phosgene poisoning the rabbit very rapidly compensates for the loss of fluid into the lungs whereas the dog does not. When the fluid stores are deprived of water for a time, however, the rabbit behaves like the dog in this respect. It is probable that if rabbits were placed on a dry diet for a few days the haemodilution after haemorrhage would also be much slower. This is at present under investigation. Courtice & Gunton (1949 *b*) have shown that an increase in water intake above normal significantly increases the plasma volume of rabbits and presumably also the available tissue fluid, so a decrease in normal water intake probably has the reverse effect.

In the rabbits studied in the experiments described above, the plasma volume is above the original level at 2 hr. when the plasma protein concentration is low. At 24 hr., when the plasma protein level is restored to its original value, the plasma volume is only slightly greater than at 2 hr. It is not until the red cells regenerate to restore the red cell volume to normal that the plasma volume falls once more to its original value. This suggests that in rabbits at least the total circulating protein is not only the controlling factor in determining the final plasma volume. It may be that the viscosity of the blood, by affecting the peripheral resistance and therefore the blood pressure, plays a part.

SUMMARY

1. The effect of nembutal anaesthesia on the restoration of fluid to the blood stream after haemorrhage has been studied in dogs, cats and rabbits.

2. In unanaesthetized dogs and rabbits and in anaesthetized dogs, cats and rabbits, there is no evidence of splenic contraction to increase the circulating blood volume after non-fatal haemorrhage.

3. Nembutal anaesthesia inhibits the restoration of the plasma volume after haemorrhage in dogs and cats and slows down this process in rabbits.

4. Rabbits behave differently from dogs in that they very rapidly over-compensate by withdrawal of fluid from the tissues.

We are indebted to Mr P. J. Phipps for technical assistance.

REFERENCES

Barcroft, J., Harris, H. A., Orahovats, D. & Weiss, R. (1925). *J. Physiol.* **60**, 443.
Bollman, J. L., Svirbley, J. & Mann, F. C. (1938). *Surgery*, **4**, 881.
Cameron, G. R. & Courtice, F. C. (1946). *J. Physiol.* **105**, 175.
Courtice, F. C. (1943). *J. Physiol.* **102**, 290.
Courtice, F. C. & Gunton, R. W. (1949 *a*). *J. Physiol.* **108**, 142.
Courtice, F. C. & Gunton, R. W. (1949 *b*). *J. Physiol.* **108**, 405.
Ebert, R. V., Stead, E. A. & Gibson, J. G. (1941). *Arch. intern. Med.* **68**, 578.
Hahn, P. F., Bale, W. F. & Bonner, J. F. (1943). *Amer. J. Physiol.* **138**, 415.
Izquierdo, J. J. & Cannon, W. B. (1928). *Amer. J. Physiol.* **84**, 545.
Jarcho, L. W. (1943). *Amer. J. Physiol.* **138**, 458.
Nylin, G. (1947). *Amer. J. Physiol.* **149**, 180.
Wiggers, C. J. (1944). *Physiology in Health and Disease*, 4th ed., p. 349. London: Kimpton.

J. Physiol. (1949) 108, 427–439 577·155·3·547·233:612·146

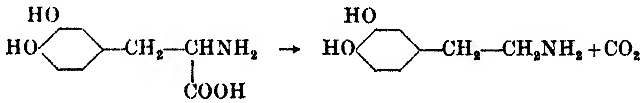
ENZYMIC FORMATION OF PRESSOR AMINES

By H. BLASCHKO, PAMELA HOLTON and G. H. SLOANE STANLEY

From the Department of Pharmacology, University of Oxford

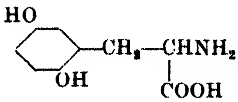
(*Received* 7 *July* 1948)

Pressor amines can be formed by the decarboxylation of amino-acids. Dopa decarboxylase is a mammalian enzyme which catalyses a reaction of this type, the formation of β-(3 : 4-dihydroxyphenyl)-ethylamine from the L-form of β-(3 : 4-dihydroxyphenyl)-alanine (Holtz, Heise & Lüdtke, 1938):

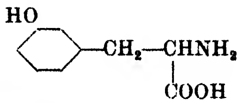


3:4-Dihydroxyphenylalanine 3:4-Dihydroxyphenylethylamine

Two new substrates of the enzyme are described in this paper, namely, 2 : 5-dihydroxyphenylalanine and meta-tyrosine (*m*-hydroxyphenylalanine). Both these amino-acids give rise to pressor amines on decarboxylation, and some of the properties of these amines are also described.



2:5-Dihydroxyphenylalanine



Meta-tyrosine
(*m*-hydroxyphenylalanine)

MATERIAL AND METHODS

The preparation of the tissue extracts and the plan of the manometric experiments differed in no way from the methods already described (Blaschko, 1942). Two organs known to contain dopa decarboxylase were used, the rat's liver and the guinea-pig's kidney. The latter organ gives extracts with high enzymic activity. The amino-acids were incubated with the tissue extracts in an atmosphere of nitrogen at a temperature of 37·5°.

The pressor action of the amines formed was studied on the arterial blood pressure of the spinal cat. DL-meta-tyrosine was kindly prepared by Dr H. R. Ing by the method described by Blum (1908). Meta-tyramine (*m*-hydroxyphenylethylamine) hydrochloride, prepared in the laboratory of Messrs Burroughs Wellcome and Company at Tuckahoe, N.Y., was made available through the kindness of Dr C. H. Kellaway.

2 : 5-dihydroxyphenylalanine has recently been synthesized by Dr A. Neuberger (1948). We are grateful to him for a sample of the racemic product, as well as of the two stereoisomers. He also gave us a sample of 2 : 5-dihydroxyphenylethylamine hydrochloride; this is also a new substance.

RESULTS

(1) *Observations with 2 : 5-dihydroxyphenylalanine*

(*a*) *Carbon dioxide formation from* L-2 : 5-*dihydroxyphenylalanine.* Carbon dioxide was formed when DL-2 : 5-dihydroxyphenylalanine was incubated under anaerobic conditions with tissue extracts containing dopa decarboxylase. The rate of CO_2 formation was compared with that which occurred when DL-3 : 4-dihydroxyphenylalanine was used as substrate. A typical experiment of this kind is shown in Fig. 1, in which 0·4 ml. of a M/50 solution of amino-acid was added to each flask. The rate of the reaction was slower with the 2 : 5-acid. At the end of the incubation, which lasted for 27 min., 86·5 μl. of CO_2 had been formed. At this time the reaction had come to a standstill. 86·5 μl. of CO_2 correspond to 0·48 moles CO_2 per mole of amino-acid added. This result suggests that only one of the two stereoisomers had given rise to carbon dioxide, probably that with the L configuration.

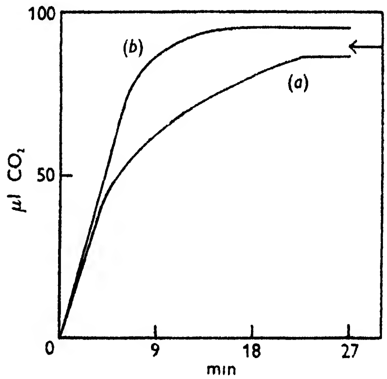


Fig. 1. Decarboxylation of 2 : 5-dihydroxyphenylalanine (*a*) and 3 : 4-dihydroxyphenylalanine (*b*) by an extract of guinea-pig's kidney. Abscissa: time in minutes; ordinate: μl. CO_2 formed. The arrow denotes half a molecule of CO_2 per molecule of DL-amino-acid added.

This was confirmed when the two stereoisomers were tested separately. Of each of the two isomers, 0·4 ml. of a M/100 solution were incubated with extract of guinea-pig's kidney. No CO_2 was formed from the dextrorotatory compound; from the laevorotatory isomer 94 μl. of CO_2 were formed, that is, 1·05 molecules of CO_2 per molecule of amino-acid added. It is known that dopa decarboxylase is specific for the L-configuration and does not act on D-3 : 4-dihydroxyphenylalanine (Holtz *et al.* 1938; Blaschko, 1942); our results therefore suggest that the laevorotatory form of 2:5-dihydroxyphenylalanine has the L-configuration. This is in agreement with the chemical evidence (Neuberger, 1948).

Dopa decarboxylase is thought to be responsible for the decarboxylation of 2 : 5-dihydroxyphenylalanine for these reasons:

(1) Decarboxylation of the 2 : 5-acid occurs in extracts of both rat's liver and guinea-pig's kidney, organs known to contain the enzyme.

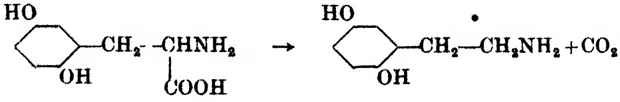
(2) The ratio

$$\frac{q_{CO_2}^{3:4\text{-acid}}}{q_{CO_2}^{2:5\text{-acid}}}$$

is very similar for both organs; in each the reaction is somewhat slower with the 2 : 5-acid.

(3) The rates of carbon dioxide formation, with both acids added together, were not additive; in one experiment with guinea-pig's kidney extract the amounts of CO_2 formed in 6 min. were: with M/500 L-2 : 5-dihydroxyphenylalanine, 25 μl.; with M/125 L-3 : 4-dihydroxyphenylalanine, 25·5 μl.; with both acids, 28 μl.

(b) *The pressor action of β-(2 : 5-dihydroxyphenyl)-ethylamine.* The experiments described above suggested that L-2 : 5-dihydroxyphenylalanine reacted in the tissue extracts, according to the equation:



L-2:5-Dihydroxyphenylalanine 2:5-Dihydroxyphenylethylamine

The product of decarboxylation is 2 : 5-dihydroxyphenylethylamine, a substance which had not been made by the organic chemist at the time when our first experiments were carried out. Since the amine was expected to have sympatheticomimetic properties, it was thought desirable to compare the activity of the substance formed by enzymic action with that of the synthetic product. Leaf & Neuberger (1948) prepared 2:5-dihydroxyphenylethylamine and we are indebted to them for the sample used in the following experiments.

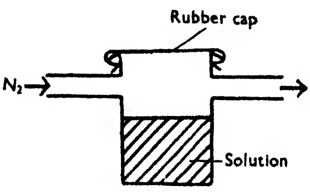


Fig. 2. Assay vessel. Half size. The vessel is closed by a rubber cap and nitrogen is passed through.

In aqueous solutions 2 : 5-dihydroxyphenylethylamine is easily oxidized, and it was necessary to protect the amine from oxidation during the course of the assay. The solutions containing the amine were kept in a glass vessel shown in Fig. 2. A rubber membrane was tied over the top and a slow stream of nitrogen was passed through the vessel. A weight was put on top of the rubber cap; this was removed when the needle attached to a syringe was plunged through the membrane in order to remove some of the solution for an injection.

Fig. 3 shows a record of the arterial blood pressure of the spinal cat. The effect of an intravenous injection of the 2 : 5-amine was compared with that of an equal amount of the corresponding 3 : 4-dihydroxyphenylethylamine. One mg. of each amine was given. The effect with each substance was a blood-pressure rise, but the type of response was different. After the injection of the 2 : 5-amine the rise was less steep and the maximum attained was less than that after an injection of the 3 : 4-amine. The rise of blood pressure due to the 3 : 4-amine was more reminiscent of an injection of adrenaline. The duration of the blood-pressure rise was more prolonged with the 2 : 5-amine.

The greater similarity of the blood-pressure response of the 3 : 4-amine to that of adrenaline is well shown on Fig. 4A, in which approximately equi-pressor doses of adrenaline and the two dihydroxyphenylethylamines were given. The different type of response to the 2 : 5-amine is evident.

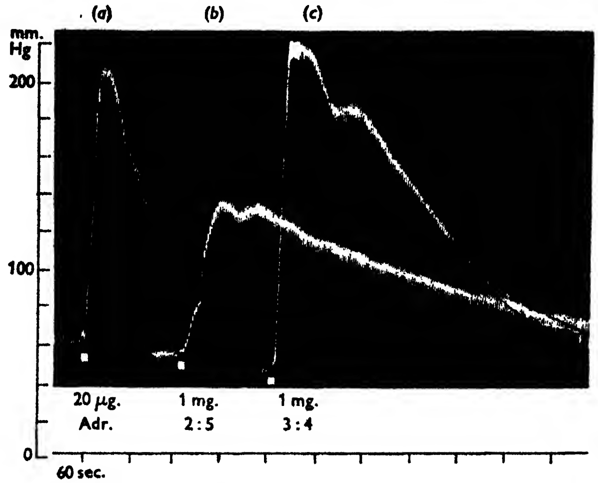


Fig. 3. Spinal cat; arterial blood pressure. The effect of intravenous injection of: (*a*) 20 µg. adrenaline; (*b*) 1 mg. 2 : 5-dihydroxyphenylethylamine (synthetic); (*c*) 1 mg. 3 : 4-dihydroxyphenylethylamine (synthetic).

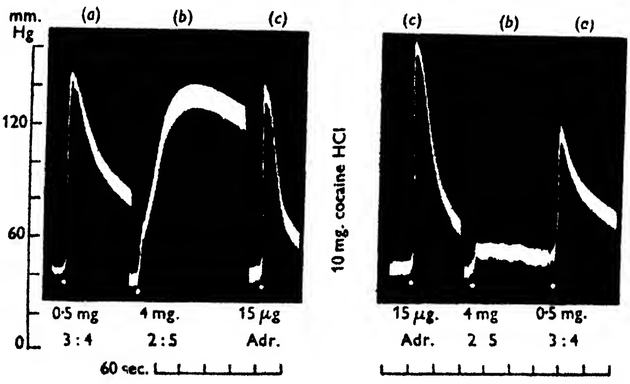


Fig. 4A. Fig. 4B.

Fig. 4. Spinal cat; arterial blood pressure. Fig. 4A shows the pressor actions on intravenous injection of: (*a*) 0·5 mg. 3 : 4-dihydroxyphenylethylamine (synthetic); (*b*) 4 mg. 2 : 5-dihydroxyphenylethylamine (synthetic); (*c*) 15 µg. adrenaline. Fig. 4B shows the action of the same doses, after an intravenous injection of 10 mg. cocaine hydrochloride.

In the same experiment 10 mg. of cocaine hydrochloride were injected. Fig. 4B shows that the effect of adrenaline was enhanced, that of the 3 : 4-amine slightly reduced and that of the 2 : 5-amine was almost abolished. In another experiment of this kind the result with the 2 : 5-amine was essentially

the same as that shown on Fig. 4, but the blood-pressure rise after the injection of the 3 : 4-amine was the same before and after cocaine.

In another experiment 2·5 mg. of ergotoxine ethanesulphonate were injected intravenously. Fig. 5 shows that the pressor action of the 2 : 5-amine was reversed.

(*c*) *Assay of pressor activity produced by enzyme action on* 2 : 5-*dihydroxy-phenylalanine*. For this experiment three manometric flasks were set up, each containing in the main vessel 1·0 ml. of guinea-pig's kidney extract (corresponding to 0·5 g. of fresh tissue) and 0·6 ml. of M/15 sodium phosphate buffer

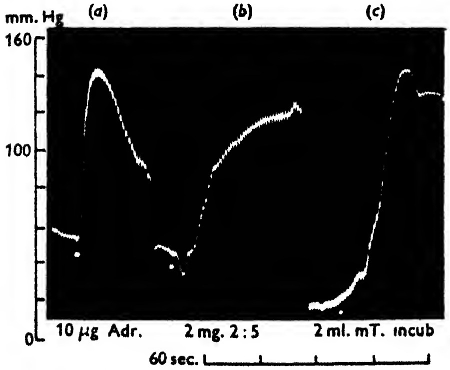


Fig. 5 A.

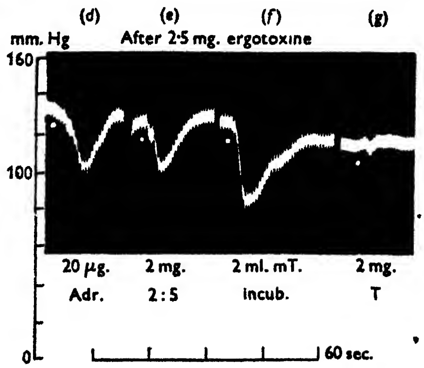


Fig. 5 B.

Fig. 5. Spinal cat; record of arterial blood pressure. Fig. 5 A. The effect of intravenous injection of: (*a*) 10 μg. adrenaline; (*b*) 2 mg. 2 : 5-dihydroxyphenylethylamine (synthetic); (*c*) 2 ml. of guinea-pig's kidney extract containing meta-tyramine produced by enzyme action. Fig. 5 B. The effect of intravenous injection, after 2·5 mg. ergotoxine ethanesulphonate, of: (*d*) 20 μg. adrenaline; (*e*) same as (*b*); (*f*) same as (*c*); (*g*) 2 mg. tyramine.

(pH 7·4). The side bulb of the vessels contained 0·4 ml. of M/12·5 DL-2 : 5-dihydroxyphenylalanine. The amino-acid was added to the extracts after temperature equilibrium had been attained. The incubation was carried out in nitrogen at 37·5°. After 90 min., when the reaction had almost ceased, a total of 1008 μl. of carbon dioxide had been formed in the three flasks, as compared with a theoretical total formation of 1080 μl. The contents of the manometer flasks were then rapidly transferred into the same assay vessel (shown in Fig. 2). Each flask was washed once with 0·5 ml. of distilled water and the washings were added, giving a total volume of 7·5 ml. The amount of amine expected to be present in 2 ml. of the final solution was

$$\frac{1008 \times 153 \times 2}{22400 \times 7{\cdot}5} = 1{\cdot}84 \text{ mg.},$$

the molecular weight of the base being 153.

The assay is shown on Fig. 6A. The final solution of the incubated extract had in 2 ml. a pressor action equivalent to more than 1·5 mg. and less than 2·0 mg. of the synthetic 2 : 5-dihydroxyphenylethylamine.

The degree of accuracy to be expected in an experiment of this kind is not very high, as the untreated tissue extracts cause a progressive deterioration of the spinal preparation; an injection of the tissue extract alone was without pressor response.

Fig. 6B shows that the pressor response in the incubated extracts was abolished after cocaine, as was the response to the synthetic 2 : 5-amine.

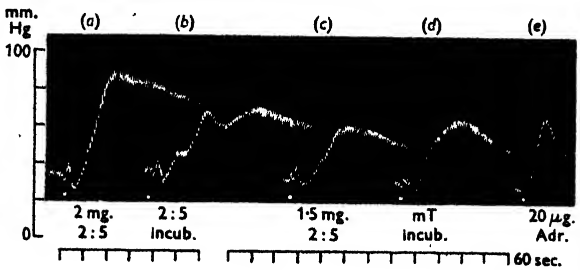


Fig. 6 A.

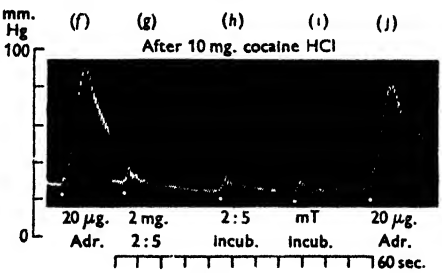


Fig. 6 B.

Fig. 6. Spinal cat; the effect on arterial blood pressure of intravenous injection of—Fig. 6A (*a*) 2 mg. 2 : 5-dihydroxyphenylethylamine (synthetic); (*b*) guinea-pig's kidney extract expected to contain 1·84 mg. 2 : 5-dihydroxyphenylethylamine; (*c*) 1·5 mg. 2 : 5-dihydroxyphenylethylamine; (*d*) meta-tyramine produced by enzyme action; (*e*) 20 μg. adrenaline. Fig. 6B, after 10 mg. cocaine hydrochloride: (*f*) same as (*e*); (*g*) same as (*a*); (*h*) same as (*b*); (*i*) same as (*d*); (*j*) same as (*e*).

(*d*) 2 : 5-*dihydroxyphenylalanine and amino-acid oxidases.* Experiments on 2 : 5-dihydroxyphenylalanine as substrate of oxidizing enzymes are not easy, since aqueous solutions of the amino-acid are not very stable under aerobic conditions. We have not been able to satisfy ourselves that the DL-form of the amino-acid was oxidized by an acetone-dried preparation of pig's kidney which had good D-amino-acid oxidase activity. Since it is known that both L-tyrosine and L-3 : 4-dihydroxyphenylalanine are substrates of the ophio-L-amino-acid oxidase (Zeller & Maritz, 1944), we have incubated the 2 : 5-acid with cobra

venom in oxygen: whereas both L-tyrosine and L-3 : 4-dihydroxyphenylalanine were rapidly oxidized, there was practically no oxygen uptake with DL-2 : 5-dihydroxyphenylalanine.

(*e*) 2 : 5-*dihydroxyphenylethylamine as substrate of amine oxidase.* Difficulty was also encountered when the 2 : 5-amine was examined as a substrate of oxidation. The solution of the amine was prepared immediately before use and added to the side bulb of the manometer flasks after these had been mounted and during the gassing with oxygen; the time allowed for temperature equilibration was reduced to 10 min. An acetone-dried powder of rabbit's liver served as source of the enzyme; 40 mg. of this powder in 1·6 ml. of M/15 sodium phosphate buffer (pH 7·4) was used. Six manometer flasks were set up; all contained the enzyme suspension plus 0·2 ml. of a neutralized M/50 solution of potassium cyanide. In the last three flasks two drops of octyl alcohol were added. The side bulbs of the three vessels contained 0·2 ml. of water, 0·2 ml. of M/20 tyramine hydrochloride or 0·2 ml. of M/20 2 : 5-dihydroxyphenylethylamine hydrochloride respectively. Under these conditions the oxygen uptake in the presence of tyramine was completely inhibited by octyl alcohol. The oxygen uptake with the 2 : 5-amine in the first two 3 min. intervals was approximately halved in the presence of octyl alcohol. At the end of the incubation, which was continued for 100 min., the total oxygen uptake in the absence of octyl alcohol was 140 μl.; in the presence of octyl alcohol it was 94 μl. In this flask the oxygen consumption had come to a standstill; in the flask without octyl alcohol the oxygen uptake between the 80 and 100 min. readings was 2·5 μl. The contents of both flasks which had contained the amine were darker than those of the blanks at the end of the incubation, but this darkening was less marked in the flask which contained octyl alcohol.

It is known that amine oxidase is cyanide-insensitive, but inhibited by octyl alcohol. The experiment described indicates that the oxygen uptake in the presence of cyanide was partly due to amine oxidase; this part of the oxygen uptake was abolished in the presence of octyl alcohol.

(2) *Observations with meta-tyrosine*

(*a*) *Formation of carbon dioxide.* Only the racemic form of meta-tyrosine was at our disposal. Formation of carbon dioxide occurred when the amino-acid was added to extracts of guinea-pig's kidney or rat's liver. In one experiment, in which 1·2 ml. of guinea-pig's kidney extract (equivalent to 600 mg. of fresh weight of tissue) were used, 79 μl. of CO_2 were formed in 9 min. from 0·4 ml. of M/50 DL-meta-tyrosine added. This corresponds to 0·44 moles of CO_2 per mole of amino-acid added. This and all our subsequent experiments suggest that only the amino-acid with the L-configuration was a substrate of the enzyme.

The evidence that dopa decarboxylase was responsible for the decarboxylation of meta-tyrosine is the same as for 2 : 5-dihydroxyphenylalanine. The

reaction was catalysed by extracts of organs which contain this enzyme; the q_{CO_2} ratios in rat's liver and guinea-pig's kidney extracts were similar, as shown in Table 1. In an experiment, in which the 3 : 4-acid and meta-tyrosine were added together, the rate of decarboxylation was approximately the same as with either substrate alone.

TABLE 1. Rates of decarboxylation of 3:4-dihydroxyphenylalanine and meta-tyrosine by tissue extracts

Organ	$q_{CO_2}^{3:4\text{-acid}}$ (A)	$q_{CO_2}^{\text{meta-tyrosine}}$ (B)	Ratio: (A)/(B)
Guinea-pig's kidney	1·70	1·58	1·08
Rat's liver	0·65	0·55	1·17

(*b*) *Assay of pressor activity produced by incubating meta-tyrosine with guinea-pig's kidney extract.* Two manometer vessels, each containing 1·0 ml. of guinea-pig's kidney extract, 0·6 ml. of M/15 sodium phosphate buffer (pH 7·4) and 0·4 ml. of a M/12·5 solution of DL-meta-tyrosine were incubated anaerobically for 1 hr.; after this time the formation of CO_2 had come to a standstill. The formation of carbon dioxide did not quite reach the theoretical amount of 358 μl.; the reaction stopped after about 300 μl. of CO_2 had been formed. Each vessel was washed with 0·2 ml. of distilled water, and the extracts and washings from both flasks were pooled in a small centrifuge tube. Unlike the 2 : 5-amine, meta-tyramine is not destroyed by heating, and the centrifuge glass was therefore placed in boiling water for 5 min. The resulting protein precipitate was spun down on a centrifuge, and the supernatant fluid was used in the assay. The result of the assay is shown on Fig. 7. From the amount of CO_2 formed we expected 2 ml. to contain 1·67 mg. of meta-tyramine; the tracings show that the amount found was equivalent to more than 1 mg., and less than 2 mg., of tyramine. The extract without added meta-tyrosine, which had served as the blank in the manometric experiment, was also boiled. The supernatant fluid was tested and found to be without pressor effect.

This experiment was repeated after synthetic meta-tyramine had become available. In the manometric experiment the CO_2 formation was 321 μl., as compared with the theoretical figure of 358 μl. Fig. 8 shows the blood pressure response to 1 mg. of meta-tyramine, compared with the response to the extract boiled after incubation with meta-tyrosine. The amount of meta-tyramine expected to be formed is 0·9 mg., if the figure of 321 μl. of CO_2 is used in the calculation; if the theoretical figure of 358 μl. of CO_2 is used the amount expected is 1·0 mg. This shows that both size and shape of the blood-pressure response with the amine produced by enzyme action are those of the synthetic meta-tyramine.

(*c*) *Pharmacological properties of meta-tyramine.* The properties of meta-tyramine have not been studied since Barger & Dale (1910) found it to be approximately equi-active with tyramine. This is confirmed by the experiment

reported in the preceding section. After cocaine, the pressor action of meta-tyramine was abolished (Fig. 6); in this respect, therefore, meta-tyramine does not differ from tyramine.

Meta-tyramine differs from tyramine in its action on the blood pressure after a dose of ergotoxine. Fig. 5 shows a reversal of the blood-pressure action of meta-tyramine after ergotoxine, whereas the action of tyramine was abolished. In this experiment we used meta-tyramine produced by enzyme action; we have since repeated the experiment with synthetic meta-tyramine; although the reversal was not as marked as in the experiment of Fig. 5, a reversal of the blood-pressure effect after ergotoxine was also seen with the synthetic meta-tyramine.

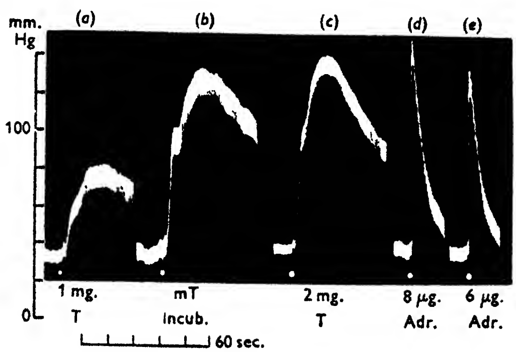


Fig. 7.

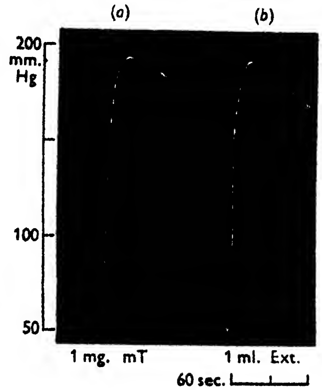


Fig. 8.

Fig. 7. Spinal cat: arterial blood pressure. Assay of meta-tyramine produced by enzyme action. Effect of intravenous injection of: (*a*) 1 mg. tyramine; (*b*) 2 ml. boiled guinea-pig's kidney extract (calculated to contain 1·67 mg. of meta-tyramine); (*c*) 2 mg. tyramine; (*d*) 8 μg. adrenaline; (*e*) 6 μg. adrenaline.

Fig. 8. Spinal cat; arterial blood pressure. Meta-tyramine produced by enzyme action compared with synthetic meta-tyramine. Effect of intravenous injection of: (*a*) 1 mg. meta-tyramine (synthetic); (*b*) 1 ml. of guinea-pig's kidney extract incubated with meta-tyrosine (expected to contain 0·9 mg. of meta-tyramine).

(*d*) *Meta-tyrosine as a substrate of amino-acid oxidases.* DL-Meta-tyrosine was found to be a substrate of both L-amino-acid oxidase and D-amino-acid oxidase. In an experiment with ophio-oxidase (3·2 mg. of cobra venom in 1·6 ml. of M/15 sodium phosphate buffer of pH = 7·4 and 0·4 ml. of a M/50 solution of DL-meta-tyrosine), there was an uptake of 78·5 μl. of oxygen in 14 min., after which time the reaction came to a standstill. This corresponds to about 0·9 moles of oxygen per mole of substrate added.

For the experiment on D-amino-acid oxidase we used an acetone-dried powder of pig's kidney (2 g. of powder extract with 20 ml. of M/15 sodium

phosphate buffer). Of this extract, 0·6 ml. was incubated with 1·4 ml. of a M/25 solution of DL-meta-tyrosine. There occurred a rapid uptake of oxygen, 40 μl. of O_2 being consumed within the first 15 min. The reaction was not followed to completion.

(*e*) *Meta-tyramine as substrate of amine oxidase.* We find that meta-tyramine is a substrate of amine oxidase. This is in agreement with Randall (1946), who used fresh extracts of guinea-pig's and cat's liver as source of the enzyme. We incubated an acetone-dried preparation of rabbit's liver with both tyramine and meta-tyramine in the presence of oxygen and we found that the rate of oxygen uptake was about the same with both amines.

DISCUSSION

Our experiments suggest that both L-meta-tyrosine and L-2 : 5-dihydroxyphenylalanine are substrates of dopa decarboxylase. This observation allows us to formulate the conditions governing substrate specificity of the enzyme with greater precision than has so far been possible. Most of the information available was related to the side chain of the molecule: it is known that on the introduction of an *N*-methyl group or of a hydroxyl group in the β position, the compounds are no longer acted on by the enzyme (Blaschko, Holton & Sloane Stanley, 1948; Blaschko, 1942).

As to the importance of the ring structure, it is known that tyrosine is not a substrate of dopa decarboxylase. The substrate specificity of the enzyme must therefore be dependent either upon the number of phenolic hydroxyl groups or on the presence of a hydroxyl group in a given position. Our results show that, irrespective of the number of hydroxyl groups, all three amino-acids with the hydroxyl group in the meta-position are substrates of the enzyme. The presence of the hydroxyl group in the para-position is not essential.

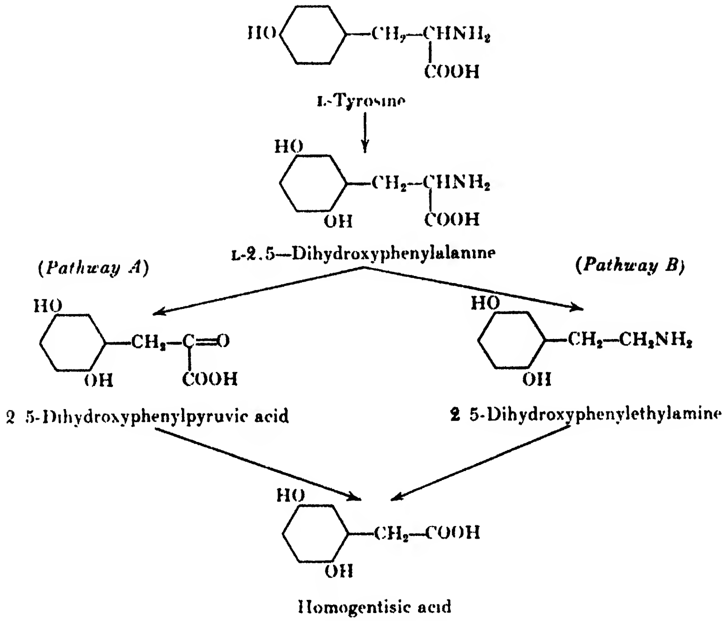
Are the two decarboxylation reactions described in this paper of physiological importance? There are indications that 2 : 5-dihydroxyphenylalanine may occur in the breakdown of tyrosine. This follows from observations on alkaptonuria, a metabolic disorder in which another 2 : 5-dihydroxy derivative, homogentisic acid, is excreted. Homogentisic acid is considered a normal product of tyrosine metabolism in man. Pathways of homogentisic acid formation from tyrosine were discussed by Neubauer (1928); two of these pathways involved the occurrence of 2 : 5-dihydroxyphenylalanine as an intermediate metabolite.

Neuberger, Rimington & Wilson (1947) have recently shown that 2 : 5-dihydroxyphenylalanine is a possible precursor of homogentisic acid. They found that in a case of human alkaptonuria at least 40% of the ingested DL-form of 2 : 5-dihydroxyphenylalanine was converted into homogentisic acid. They suggested that with the L-form the percentage conversion would be much higher, and they considered the possibility of the L-isomer as a normal intermediate between L-tyrosine and homogentisic acid.

Such an assumption would require that the D-form of the 2 : 5-acid does not significantly contribute to the formation of homogentisic acid. One would therefore expect:

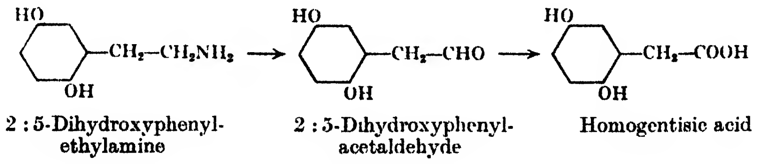
(1) the breakdown of the 2 : 5-acid to be catalysed by an enzyme specific for the L-configuration; and,

(2) the inversion of the D-isomer in the body to be slow.



Scheme 1. Pathways of formation of homogentisic acid.

Our results show that pathway (*B*) fulfils the first of these conditions. It has been established that the L-isomer is decarboxylated, whereas the corresponding D-isomer is not. We have also shown that the amine formed is a substrate of amine oxidase. Therefore, the amine would be further metabolized to the corresponding aldehyde which, on further oxidation, would give homogentisic acid:



Scheme 2. Formation of homogentisic acid from 2 : 5-dihydroxyphenylethylamine.

In their discussion of homogentisic acid formation Neuberger *et al.* (1947) suggest that L-2 : 5-dihydroxyphenylalanine is further metabolized by oxidative deamination (Pathway (*A*) of Scheme 1). This is the more orthodox path of

amino-acid breakdown in mammals; it also requires stereospecific enzymes as catalysts. It remains, however, to be shown that 2 : 5-dihydroxyphenylalanine is a substrate of amino-acid oxidases. The L-form of the acid, unlike those of tyrosine, meta-tyrosine and 3 : 4-dihydroxyphenylalanine, is not a substrate of the L-amino-acid oxidase of cobra venom; experiments with mammalian enzymes were not conclusive.

More recently, on the basis of observations in alkaptonuria, Neuberger (1948) also considers pathway (*B*) as more likely than pathway (*A*).

In their first paper on dopa decarboxylase, Holtz *et al.* (1938) discussed the possibility that a decarboxylation reaction was a normal occurrence in the breakdown of L-amino-acids and preceded oxidative deamination; they suggested that L-amino-acid oxidase might not be one enzyme but might consist of two: an amino-acid decarboxylase plus an amine oxidase. This possibility was put to an experimental test and not confirmed when it was found that the decarboxylation reaction was specific for 3 : 4-dihydroxyphenylalanine and that other amino-acids were not metabolized in the same way. The interpretation given to the substrate specificity of dopa decarboxylase was that the enzyme might catalyse an intermediate step in the biosynthesis of adrenaline and sympathin (Blaschko, 1939, 1942, 1949). The experiments described in this paper show that dopa decarboxylase may have another role in the metabolism of tyrosine.

There is no previous study of 2 : 5-dihydroxyphenylethylamine, but the corresponding *N*-methyl derivative has been made by Buck (1932) and its pharmacological actions have been briefly described (Hjort, 1934). Like the primary amine, the secondary amine proves less active on the blood pressure after cocaine, but Hjort (1934) did not observe a reversal of the pressor effect after ergotoxine with the *N*-methylated amine.

As far as meta-tyrosine is concerned, there is no record of its occurrence in mammals; *m*-hydroxybenzoic acid has been found by Lederer (1941) in the dried scent glands of the Canadian beaver (*Castor fiber* L.). Since meta-tyramine is a substrate of amine oxidase it is conceivable that *m*-hydroxybenzoic acid is derived from meta-tyrosine.

A comparison of the substrate specificity of dopa decarboxylase with that of the L-tyrosine decarboxylase of *Streptococcus faecalis* shows that the bacterial enzyme differs from the mammalian enzyme not only in its high affinity for tyrosine, but also in its low affinity for 2 : 5-dihydroxyphenylalanine. Recent experiments have shown (Sloane Stanley, unpublished) that the bacteria are unable to decarboxylate 2 : 5-dihydroxyphenylalanine; meta-tyrosine is decarboxylated by an acetone-dried preparation of the bacteria.

SUMMARY

1. L-2 : 5-Dihydroxyphenylalanine and L-meta-tyrosine (*m*-hydroxyphenylalanine) are decarboxylated in extracts of guinea-pig's kidney and of rat's liver; it is likely that these reactions are catalyzed by the enzyme dopa decarboxylase.

2. The amines formed in the decarboxylation reaction are 2 : 5-dihydroxyphenylethylamine and meta-tyramine (*m*-hydroxyphenylethylamine); they have pressor action, and the amounts of amine formed in the decarboxylation reaction have been determined by assay on the arterial blood pressure of the spinal cat. The pressor action of 2 : 5-dihydroxyphenylethylamine is reversed after ergotoxine; it is decreased after cocaine. The pressor action of meta-tyramine is abolished by cocaine; after ergotoxine, the pressor action of meta-tyramine is reversed.

3. The decarboxylation of 2 : 5-dihydroxyphenylalanine is of interest in connexion with the formation of homogentisic acid in alkaptonuria.

4. The common feature in the three substrates of dopa decarboxylase examined is the hydroxyl group in the meta-position to the side chain.

5. Both 2 : 5-dihydroxyphenylethylamine and meta-tyramine are substrates of amine oxidase.

6. Meta-tyrosine is oxidized by D-amino-acid oxidase of pig's kidney and by the L-amino-acid oxidase of cobra venom; 2 : 5-dihydroxyphenylalanine does not appear to be oxidized by the cobra venom enzyme.

The authors are grateful to Prof. J. H. Burn for help and advice on the pharmacological aspects of this work.

One of us (G. H. S. S.) is grateful to the Medical Research Council for a personal grant.

REFERENCES

Barger, G. & Dale, H. H. (1910). *J. Physiol.* **41**, 19.

Blaschko, H. (1939). *J. Physiol.* **96**, 50*P*.

Blaschko, H. (1942). *J. Physiol.* **101**, 337.

Blaschko, H. (1949). Adrenaline and sympathin. In *The Hormones. Physiology, Chemistry and Applications*, vol. II, chapter 25*b*. New York: Academic Press Inc.

Blaschko, H., Holton, P. & Sloane Stanley, G. H. (1948). *Biochem. J.* **42**, xlviii.

Blum, L. (1908). *Arch. exp. Path. Pharmak.* **59**, 269.

Buck, J. S. (1932). *J. Amer. chem. Soc.* **54**, 3661.

Hjort, A. M. (1934). *J. Pharmacol.* **52**, 101.

Holtz, P., Heise, R. & Lüdtke, K. (1938). *Arch. exp. Path. Pharmak.* **191**, 87.

Leaf, G. & Neuberger, A. (1948). *Biochem. J.* **43**, 606.

Lederer, E. (1941). *Bull. Soc. Chim. biol., Paris*, **23**, 1457.

Neubauer, O. (1928). *Handbuch der normalen und pathologischen Physiologie*, **5**, 860. Berlin: Springer.

Neuberger, A. (1948). *Biochem. J.* **43**, 599.

Neuberger, A., Rimington, C. & Wilson, J. M. G. (1947). *Biochem. J.* **41**, 438.

Randall, L. O. (1946). *J. Pharmacol.* **88**, 216.

Zeller, E. A. & Maritz, A. (1944). *Helv. chim. Acta*, **27**, 1888.

J. Physiol. (1949) 108, 440–450 612.533

THE CHANGES IN WATER AND CHLORIDE DISTRIBUTION DURING HEAVY SWEATING

By W. S. S. LADELL

From the Colonial Medical Research Committee Laboratory for Hot Climate Physiology, Medical School, Yaba, Lagos, Nigeria

(*Received* 20 *July* 1948)

Correlated observations on man between changes in salt and water balance and changes in the blood electrolytes during the acute salt loss that occurs during heavy sweating have not been made to the same extent as corresponding observations on animals during acute salt loss otherwise induced. Lee, Murray, Simmonds & Atherton (1941) discussed the strain which working in the heat imposes upon the salt/water balance, but they did not report any figures for the blood electrolyte concentration; their subjects were not sweating at very high rates, the maximum being 10·4 c.c./min., and the salt losses were all small, often less than 1 g. and never more than 2 g. in an 8 hr. test, considerably less than the losses in the urine over the same period. McCance's studies (1936, 1937, 1938) were on subacute rather than on acute salt deficiency, and sweating was only one of the means by which he induced the deficiency; the blood electrolytes were estimated, but, as the samples were not taken in close relation to the sweating periods, it is not possible to evaluate the effect of sweating as compared with other factors. Nadal, Pedersen & Maddock (1941) compared the effect of salt loss with that of water deprivation, and their results indicated that water is lost from the extracellular compartments in salt deprivation, and from the extra- and intracellular compartments in water deprivation; but as their deprivations were induced relatively slowly it would not be admissible to deduce from them the effects of a few hours of very profuse sweating.

Elkinton, Danowski & Winkler (1946*a*, *b*) studied acute salt deprivation in dogs induced by intraperitoneal injection of hypertonic glucose with subsequent removal of the intraperitoneal fluid. They correlated the changes in the blood chemistry and in the clinical condition of the animals with the changes in salt and water balance and calculated the movements of fluid between the intra- and extracellular compartments.

In the series of experiments reported elsewhere (Ladell 1947, 1948), and in others of a similar nature, there were a number of occasions in which the salt

and water balances were known accurately and when blood samples were taken immediately before and after two or more hours of very heavy sweating. During this time as much as 20 g. of sodium chloride were sometimes lost, while urine flow was practically suppressed (Weiner, 1945). From changes in the chloride content of the blood and plasma, and from the salt and water balance figures, sufficient data were available to calculate the fluid shifts according to the methods of Elkinton & Winkler (1944).

METHODS

Men were subjected to the routine of alternate work and rest described previously (Ladell, 1947) for periods of from 110 to 170 min. in an air-conditioned room maintained at 100° F. dry-bulb temperature and 94° F. wet-bulb. The subjects were weighed before and after each bout of work, and sweat samples were obtained at the time of each weighing from impermeable bags worn on one arm; these bags give a true sample of 'mixed body sweat' (Ladell, 1948). Any urine passed was collected. The amount of water drunk and of salt ingested, as 10% sodium chloride solution, was accurately measured. Blood samples were taken immediately before and immediately after exposure to the heat by venepuncture with the minimum of stasis. The samples were collected under oil, and true plasma was obtained by centrifuging under paraffin wax.

The chloride content of sweat and urine samples was estimated by the Whitehouse method, and the blood and plasma chloride by the Volhard method on the Folin-Wu filtrate (Peters & Van Slyke, 1932). In a number of experiments the plasma protein and the haematocrit value were estimated by Van Slyke's copper sulphate, specific gravity method (Phillips *et al.*, 1942).

The sweat loss was calculated from the change in body weight, the water uptake and the urine passed. No correction was made for respiratory exchange; the weight change due to this is less than 10 g./hr., which is negligible in comparison with the total sweat loss, and is less than could be detected over short periods. The chloride losses were calculated for each period between weighings from the sweat water loss and the chloride concentration in the sweat sample produced during that period. When water or salt was being replaced the calculated losses during one period were given to the subject for consumption during the next period, so there was always a slight lag in the replacement; this was allowed for by giving the subject a small 'advance' of salt or water or both, as the experiment required, as soon as he entered the room; this 'advance' was included in the final balance, as was the last dose given before leaving the room, but not that given as he left the room. There was usually an interval of at least 20 min. between the last dose of salt or water included in the balance and the taking of the post-exposure blood sample.

RESULTS

Actual observations. Three young male subjects, all fit and fully acclimatized to work in the heat, completed in all thirty tests under varying conditions of salt and water intake. Sweat rates varied from 17·7 c.c./min. for 137 min. (Exp. 20) to 38·2 c.c./min. for 130 min. (Exp. 2); the greatest sweat volume lost was 5497 c.c. in 165 min. (Exp. 1) and the greatest salt loss was 25·02 g. in 162 min. (Exp. 15). The replacements, where carried out, were effective; for example, in Exp. 16, the final water balance was +28 c.c. and in Exp. 27 the final salt balance was −0·99 g. In the other experiments there were considerable disturbances of the salt and water balances, which were reflected by changes in the chloride contents of the blood and plasma, and in the haematocrit readings and in the plasma protein concentration; these changes are shown in Table 1.

TABLE 1. Summary of experiments showing the changes observed in the blood chemistry (Exps. 1–10: subject LAD. Exps. 11–19: subject BRA. Exps. 20–30: subject GOL

Exp. no.	Duration of exposure (min.)	Sweat loss: Water (c.c.)	Sweat loss: Chloride (as NaCl) (g.)	Intake: Water (c.c.)	Intake: Chloride (as NaCl) (g.)	Final balance (including urine losses): Water (c.c.)	Final balance (including urine losses): Chloride (as NaCl) (g.)	Whole blood chloride: Before exposure (m.eq./l.)	Whole blood chloride: After exposure (m.eq./l.)	Plasma chloride: Before exposure (m.eq./l.)	Plasma chloride: After exposure (m.eq./l.)	Overall change in: Plasma protein (g./100 c.c.)	Overall change in: Haematocrit reading (%)
1	165	5497	21·68	4540	Nil	− 957	−21·68	82·2	76·8	97·2	91·2	—	—
2	130	4969	22·73	3100	Nil	−1869	−22·73	78·0	74·2	96·3	90·9	—	—
3	160	4835	20·73	2370	Nil	−2465	−20·73	68·4	65·1	96·0	85·8	—	—
4	165	4963	24·58	234·5	23·45	−5101	− 1·89	84·3	87·6	103·2	109·8	+2·944	+ 7·5
5	135	4726	20·84	3667	Nil	−1276	−20·93	78·9	74·4	99·0	92·1	+0·40	− 1·1
6	135	4205	20·17	4717	Nil	+ 355	−20·67	78·6	71·1	97·8	90·6	+1·04	+ 7·2
7	135	4199	22·10	4633	Nil	+ 85	−22·75	79·0	69·3	97·6	90·1	+1·40	+ 8·0
8	135	3238	16·70	3180	Nil	− 138	−16·95	77·4	70·8	104·4	97·2	+0·35	0
9	135	3574	19·11	4270	Nil	+ 546	−19·43	78·4	66·6	100·8	88·4	+0·70	+ 3·6
10	150	4296	23·20	4428	25·58	+ 87	+ 2·27	79·6	78·0	98·8	99·2	0	+ 2·7
11	162	3401	17·43	3835	Nil	+ 47·5	−18·17	78·3	66·0	93·6	87·0	—	—
12	162	3521	14·68	Nil	Nil	−3829	−15·21	80·4	80·1	99·6	102·3	—	—
13	162	4420	18·40	4723	15·63	+ 118	− 3·68	82·2	79·5	101·4	99·9	—	+ 1·1
14	162	4797	18·50	4631	Nil	+ 324	−19·35	79·2	73·3	99·0	95·4	—	+ 2·2
15	162	4933	25·02	4619	21·88	− 577	− 3·60	76·2	76·8	93·3	97·2	—	+12·0
16	162	4333	19·97	4633	36·47	+ 28*	+15·59*	84·0	88·8	96·0	96·0	+0·23	− 2·1
17	110	2651	14·22	761	12·11	−1880*	− 3·18*	80·4	75·6	100·2	96·0	+0·26	+ 3·8
18	110	2908	14·93	Nil	Nil	−3288	−16·57	75·6	78·6	101·4	104·4	+0·94	+ 6·2
19	110	2845	15·08	136	13·63	−2933*	− 2·98*	77·4	75·0	99·0	97·0	+0·56	—
20	137	2425	9·87	3054	8·43	+ 514	− 3·12	80·1	[illegible]·4	99·3	96·3	+0·54	− 1·0
21	137	2737	12·58	3478	10·73	+ 651	− 2·62	79·2	79·2	98·4	98·4	+0·14	− 1·4
22	137	2939	13·05	3647	11·81	+ 633	− 2·61	73·8	79·6	102·6	98·7	+0·22	− 1·5
23	137	3036	10·87	3564	9·67	+ 426	− 2·21	77·7	77·7	93·6	93·0	+0·36	− 4·8
24	162	4450	18·92	4405	Nil	− 139	−19·87	78·0	72·0	98·4	92·1	+0·06	+ 0·8
25	110	2961	12·58	Nil	Nil	−3001	−12·74	74·8	78·2	97·2	100·6	+0·84	+ 3·8
26	162	4476	13·32	4447	Nil	− 118	−13·79	76·8	73·2	98·2	94·2	+0·88	+ 0·8
27	162	4074	14·10	3826	14·03	− 356	− 0·99	78·0	74·4	97·8	95·4	0	+ 2·0
28	162	5032	19·83	4898	Nil	− 214	−20·34	78 9	71·4	96·3	90·0	+0·54	+ 5·8
29	162	4802	23·89	4781	21·87	− 181	− 2·41	78·0	75·2	99·4	97·0	+0·60	− 2·8
30	110	3282	13·92	Nil	Nil	−3345	−14·29	75·2	78·0	94·6	97·2	+0·80	+ 3·2

* Balances unreliable as subject vomited and absorption was defective; but vomit collected as far as possible and included in the balance.

In general the plasma chloride fell concomitantly with the development of a negative chloride balance, and it rose or remained the same when the chloride losses were made good or when water losses as well as salt losses went unrelieved. On three occasions only did the plasma chloride fall below 90 m.equiv./l. The changes in the whole-blood chloride were usually, but not invariably, in the same direction as the plasma chloride changes; on four occasions the whole-blood chloride was reduced below 70 m.equiv./l.

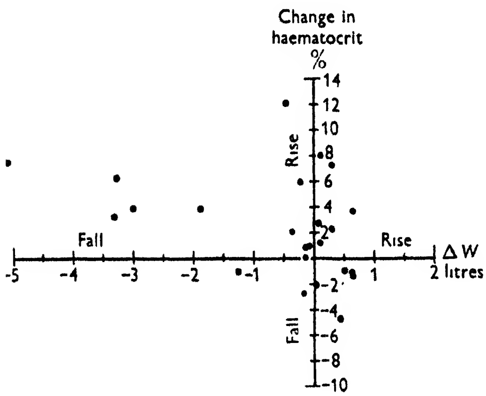


Fig. 1.

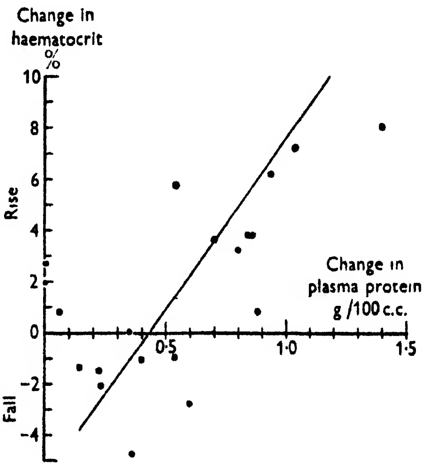


Fig. 2.

Fig. 1. Scatter diagram showing changes in haematocrit readings and changes in total body water (ΔW). No correlation was observed.

Fig. 2. Changes in haematocrit readings correlated with changes in plasma protein. Correlation coefficient, 0·671; $t = 3{\cdot}84$; P less than 0·01.

Plasma-protein changes were followed on twenty-two occasions; twice there was no change and on twenty occasions there was a rise. The changes in the haematocrit readings were not related to the changes in water balance (see Fig. 1), but were well correlated with the plasma-protein changes (see Fig. 2). Excluding two pairs of values, where the protein estimations were possibly faulty (Exps. 4 and 19) for technical reasons, there was a correlation coefficient of 0·671 ($t = 3{\cdot}84$, P less than 0·01) between the two.

Derived values. Using the method of Elkinton & Winkler (1944) the changes in extracellular fluid volume were calculated for each experiment, from the body weight, the chloride balance, and the change in plasma-chloride concentration. In Exps. 16, 17 and 19 the salt replacement by means of 10% sodium chloride was not well tolerated, the subject vomiting, and subsequently having saline diarrhoea; accurate chloride balances were not, therefore, available for these experiments. The changes in the extracellular fluid volumes calculated for the remaining twenty-seven tests are shown in Table 2. From the change

in the total body water, i.e. the water balance (ΔW), and the change in the extracellular fluid volume (ΔE), the change in the intracellular fluid volume was obtained by difference for each experiment; these changes (ΔI) are also shown in Table 2.

TABLE 2. Changes in chloride balance, body water, extracellular fluid and intracellular fluid

Exp. no.	Calculated change in: Chloride balance (as NaCl) (g.)	Body water ΔW (l.)	Extracellular fluid ΔE (l.)	Intracellular fluid ΔI (l.)
	(a) Chloride losses not replaced			
1	−21·68	−0·957	−2·936	+1·979
2	−22·73	−1·869	−2·257	+0·388
3	−20·73	−2·465	−2·137	−0·328
5	−20·93	−1·276	−2·620	+1·344
6	−20·67	+0·355	−2·618	+2·973
7	−22·75	+0·085	−2·871	+2·966
8	−16·95	−0·138	−1·778	+1·640
9	−19·43	+0·546	−1·583	+2·129
11	−18·17	+0·047	−2·110	+2·157
12	−15·21	−3·829	−2·924	−0·905
14	−19·35	+0·324	−2·708	+3·032
18	−16·57	−3·288	−3·179	−0·019
24	−19·87	−0·139	−2·285	+2·146
25	−12·74	−3·001	−2·763	−0·238
26	−13·79	−0·118	−1·528	+1·410
28	−20·34	−0·214	−2·447	+2·233
30	−14·29	−3·345	−2·871	−0·474
	(b) Chloride losses replaced			
4	− 1·89	−5·101	−1·247	−3·854
10	+ 2·27	+0·087	+0·321	−0·234
13	− 3·68	+0·118	−1·659	+1·777
15	− 3·60	−0·477	−0·227	−0·250
20	− 3·12	+0·514	+0·055	+0·459
21	− 2·62	+0·651	−0·445	+1·096
22	− 2·61	+0·633	+0·310	+0·323
23	− 2·21	+0·426	−0·272	+0·698
27	− 0·99	−0·356	+0·311	−0·667
29	− 2·41	−0·181	+0·058	−0·239

There was no direct relationship between ΔW and ΔE. The ΔI values, however, fell into two groups, depending on whether the chloride losses had been replaced or not; with replacement the mean ΔI was +40 c.c. and without replacement +1450 c.c.; this difference is significant, t being 9·85 and P less than 0·01. In both cases ΔI and ΔW ran together (see Fig. 3); significant regression and correlation coefficients were obtained for both groups, and from these regressions it appeared that when chloride was not replaced ΔI remained positive until ΔW exceeded 2·7 l.; the regression passed very near to the origin, however, when there was replacement.

The changes in plasma-chloride concentration were correlated with ΔI (Fig. 4); the correlation coefficient was −0·65 (t = 4·31, P less than 0·01). From the changes in the whole blood and in the plasma-chloride concentrations, and

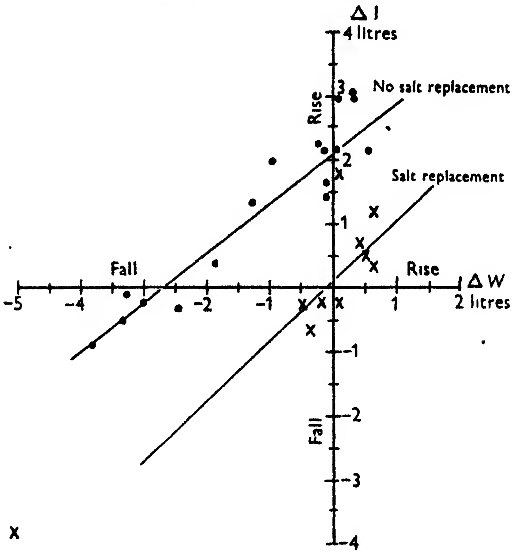


Fig. 3. The changes in intracellular fluid volume (ΔI) correlated with changes in total body water (ΔW), when salt was not replaced (spots) and when salt was replaced (crosses). Both regressions are significant, with correlation coefficients of 0·865 ($t=6·65$, P less than 0·01) and 0·807 ($t=3·86$, P less than 0·01) respectively.

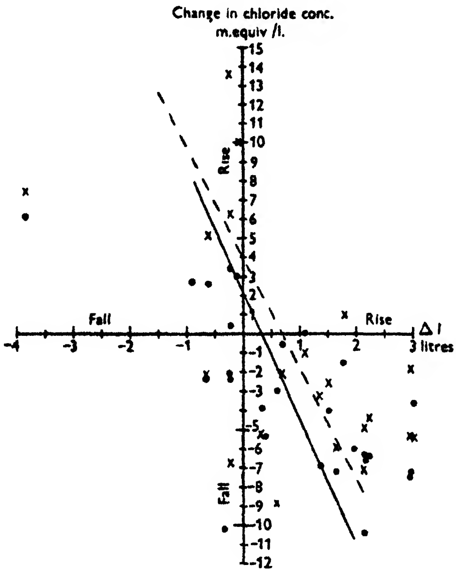


Fig. 4. Changes in the chloride content of the plasma (spots and solid line) and of the red blood cells (crosses and broken line) correlated with the changes in the intracellular fluid volume (ΔI). Both regressions are significant: for plasma correlation coefficient is $-0·65$, $t=4·31$; for cells coefficient is $-0·53$, $t=2·84$; P less than 0·01 and 0·01 respectively.

from the haematocrit values before and after exposure to the heat, the chloride concentration in the cells was calculated. The mean fall in twenty-two experiments was 4·45 m.equiv./l.; the mean fall in plasma chloride for the same series was 3·21 m.equiv./l. The cell chloride change was also correlated with ΔI, a coefficient of $-0{\cdot}53$ ($t=2{\cdot}84$, $P=0{\cdot}01$) was found. The scatter and the regression is shown in Fig. 4.

DISCUSSION

Fluid movements. Although the changes in the concentration of only one ion have been followed in these thirty experiments, the results indicate in what direction and to what extent transfer of water takes place between the extra- and intracellular compartments when men sweat heavily. The immediate source

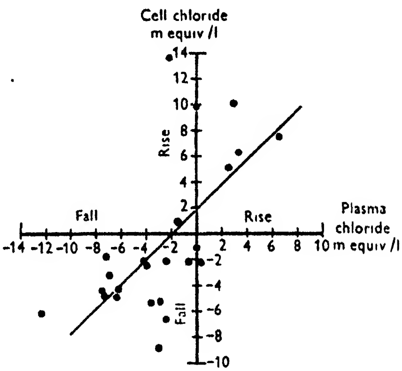


Fig. 5. Changes in the chloride content of the red blood cells correlated with the changes in the chloride content of the plasma. Correlation coefficient 0·67, $t=4{\cdot}24$, P less than 0·01.

of the water lost in the sweat must be the extracellular fluid, which also provides the chloride; depending upon the extent to which these losses are made good there might be expected a greater or less disturbance of the osmotic equilibrium between the extra- and intracellular compartments. The results of these experiments suggest that such disturbances do not develop to any great extent, the equilibrium being quickly restored by simple fluid transfer. When chloride is not replaced water flows from the extra- to the intracellular compartment, until, presumably, osmotic equilibrium is obtained; the fall in the chloride concentration of the extracellular fluid is paralleled, on the one hand by a fall in the osmotic pressure of the intracellular fluid, which is reflected by the fall in the chloride content of the blood cells (Fig. 5), and on the other hand by a rise in the intracellular fluid volume (Fig. 4). The magnitude of the fluid transfer depends upon the amount of water that is replaced and the amount of salt that is lost. In these tests the mean salt loss was 19 g.; the regression shows that, with this loss, if the subject remained in water balance the mean ΔI was +2·1 l., and with over-hydration it was greater, and that a negative water

balance of 2·7 l. had to be incurred before any water was lost from the intracellular compartment. But when salt was replaced as fully as was possible and water was either not replaced, or replaced relatively more slowly than the salt, the water loss was borne almost entirely by the intracellular compartment, and the osmotic pressure in both compartments rose, as shown by the rise in plasma and blood-cell chloride concentrations in Exps. 4, 18, 25 and 30.

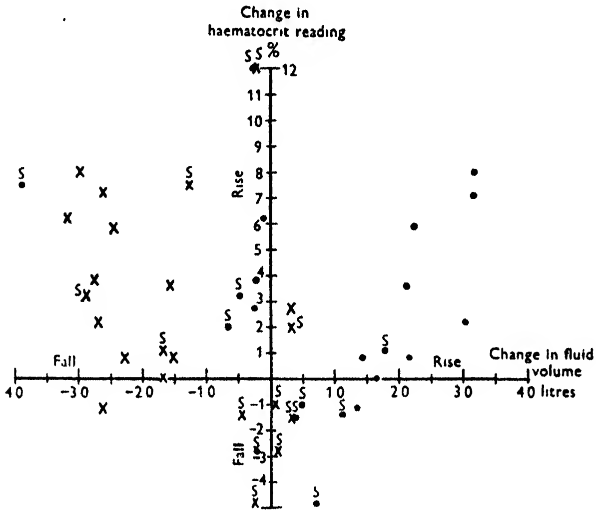


Fig. 6. Scatter diagram to show how, in general, the haematocrit reading rises as the extracellular fluid volume falls, indicated by crosses, and as the intracellular fluid volume rises, indicated by spots. There is no statistical correlation. The letter *S* above a point indicates that in that test salt was replaced.

The changes in the haematocrit readings merely reflect the relative changes in the cell and plasma volumes, and these in turn may reflect the changes in the intra- and extracellular fluid volumes. Fig. 6 shows that in general, as the intracellular fluid volume rose and the extracellular volume fell, the haematocrit reading rose; so many other factors are involved, however, that a statistical correlation was not obtainable. As no red cell counts were made the mean cell volumes could not be calculated, and so the question of the behaviour of the red blood cells as simple osmometers (McCance, 1937) remains open.

Electrolyte movements. McCance (1937) concluded that, in a salt deficiency state lasting a number of days, potassium, chloride and possibly sodium ions passed out of the cells into the plasma. The passage of base out of the cells in conjunction with cell water has also been described during water deprivation by Newburgh & Johnston (1934), who noted the rise in urinary potassium towards the end of a period of dehydration; while Johnson, Pitts & Consolazio (1944) reported a rise in the potassium content of the sweat during heavy

sweating, the source of this potassium presumably being the cells. Elkinton & Winkler (1944) considered that potassium loss from the cells was a general and compensating reversible physiological response of the organism to severe depletion of water. The passage of base out of the cells is, therefore, well established; but the movements of chloride have not been followed in such detail, nor have the movements of either base or chloride been considered during sudden and severe derangements of water and salt balance, such as are described in this communication.

In the present series of tests, if the changes in the chloride concentration in the red blood corpuscles, apart from the chloride shift, were due solely to the passage of water in and out of the cells, as part of the general transfer of fluid from the extra- to the intracellular compartments, then

$$\text{R.B.C. vol.} \times \text{R.B.C. Cl conc.} = \text{constant},$$

but
$$\text{R.B.C. vol. is proportional to I.C.F. v l.},$$

therefore
$$\text{I.C.F. vol.} \times \text{R.B.C. Cl conc.} = K \text{ (a constant)}; \tag{1}$$

differentiating gives

$$\frac{d\,(\text{I.C.F. vol.})}{d\,(\text{R.B.C. Cl conc.})} = -\frac{K}{(\text{R.B.C. Cl conc.})^2}.$$

A mean value for $\frac{d\,(\text{I.C.F. vol.})}{d\,(\text{R.B.C. Cl conc.})}$ may be obtained from the regression equation for ΔI on cell chloride:

$$\Delta I = 627{\cdot}565 - 140{\cdot}107 \text{ (change in R.B.C. Cl conc.)},$$

and by substituting this and the mean value for (R.B.C. Cl conc.), the value of K was obtained, and thence, from equation (1) and the mean (R.B.C. Cl conc.), a value for intracellular fluid volume. The figure calculated in this way was 37·85 l. This figure is a little high (cf. Marriott, 1947), but in view of the approximations involved, especially that of treating the relationships as linear, it may be considered a good result; it may therefore be concluded that, if the changes in the chloride concentration in the red blood cells may be taken as an example, the observed changes in the fluid distribution and in the chloride concentrations during a short period of severe sweating may be accounted for without postulating transfer of chloride ions.

General. Nadal *et al.* showed in 1941 that the peripheral circulatory failure seen in salt deficiency was due to the reduction in the extracellular fluid volume, and that in water deficiency water is lost from both compartments; Marriott (1947) also explained the clinical picture of dehydration, from whatever cause, on the basis of loss of extracellular fluid. But Gamble (1944) and Winkler, Elkinton, Hopper & Hoff (1944) have shown that the real danger and the ultimate cause of death in water deficiency is intracellular desiccation and the consequent rise in intracellular osmotic pressure. The beginnings of this were

seen in the present series of experiments; when salt was replaced fully and water either not at all or inadequately, the chloride concentrations inside the cells rose and the intracellular compartments were drawn upon for water; this shows the danger of replacing salt unless water can be replaced as well.

The intracellular over-hydration that occurs when salt is not replaced and water losses are either replaced or not very high is analogous to that described by Danowski, Winkler & Elkinton (1946) in over-hydrated dogs. These animals developed convulsions, the so-called water intoxication; it is possible that 'heat cramps' in man may be the same condition; in the long series of tests, of which the thirty here described are a small proportion only, the conditions for intracellular over-hydration were frequently fulfilled and 'heat cramps', fully developed or incipient, were a regular complaint. The water intoxication hypothesis of 'heat cramps' (Moss, 1922; Haldane, 1923; Hunt, 1912) has been doubted by Talbott (1935), but our experiments show that there is intoxication by water, though of the intracellular compartments and not, as earlier thought, of the extracellular.

Some similarity was looked for between the condition of the subjects at the end of an exposure in the climatic chamber and the clinical acute condition of type I heat exhaustion (Ladell, Waterlow & Hudson, 1944); the heat exhaustion cases did not sweat so profusely as the men in the climatic chambers, but they sweated for longer, and the falls in blood and plasma-chloride concentrations were correspondingly greater, and cramps were common; one biochemical feature was the proportionately greater fall of the whole-blood chloride concentration relative to the fall in the plasma chloride; this was shown by the reduction ratio

$$\frac{\text{Final blood Cl conc.}}{\text{Initial blood Cl conc.}} : : \frac{\text{Final plasma Cl conc.}}{\text{Initial plasma Cl conc.}},$$

the mean value for this being 0·95, and all values being below 1 except in mild cases. The mean ratio for the men in the climatic chamber was 0·9933 ($\pm$ 0·007 S.E.), but when the ratios were plotted against the final plasma-chloride concentration on eleven out of seventeen occasions when salt was not replaced the point came below or within the standard error of the arbitrary line, which Ladell *et al.* found separated the points plotted for type I from those plotted for type II cases; the equation for this line is

$$P + 75R = K,$$

where P = plasma-chloride concentration, R = reduction ratio, K = a constant value 165·4. (In the communication by Ladell *et al.* an error in the original manuscript resulted in the value of K being given incorrectly as 106·8.) There is, therefore, some similarity between the fluid and electrolytic changes in the acute condition seen in the climatic chamber, and those in the less acute, but more severe heat exhaustion, type I.

In the present series of tests exposure to the heat resulted in an increase in the protein content of the plasma, irrespective of the final water balance; this contrasts with the observation by Lee *et al.* (1941); they found a decrease in the serum-protein concentration, even with deficits of body water as high as 2%. The difference might be due to the very much higher rates of sweating in the present tests.

SUMMARY

1. The changes in the chloride concentration of the blood and plasma which occur when men sweat heavily as the result of exercise in a hot humid atmosphere are described.

2. When these changes are considered in relation to the alterations in the salt and water balance it can be shown that, if the salt losses in the sweat are not replaced, there is a transfer of water from the extracellular to the intracellular compartments.

3. These findings are discussed in the light of reports by other workers on salt and water movements in men and in animals, under varying conditions.

This work was done at the Medical Research Council Neurological Research Unit, National Hospital, Queen Square, London, under the direction of Dr E. Arnold Carmichael, to whom I am greatly indebted for advice and encouragement. I also wish to thank my subjects for their loyal co-operation in carrying out these tests.

REFERENCES

Danowski, T. S., Winkler, A. W. & Elkinton, J. R. (1946). *J. clin. Invest.* **25**, 130.
Elkinton, J. R., Danowski, T. S. & Winkler, A. W. (1946*a*) *J. clin. Invest.* **25**, 120.
Elkinton, J. R., Danowski, T. S. & Winkler, A. W. (1946*b*) *J. clin. Invest.* **25**, 130.
Elkinton, J. R. & Winkler, A. W. (1944). *J. clin. Invest.* **23**, 93.
Gamble, J. L. (1944). *Proc. Amer. Phil. Soc.* **88**, 3.
Haldane, J. S. (1923). *Brit. med. J.* i, 986.
Hunt, E. H. (1912). *J. Hyg., Camb.*, **12**, 479.
Johnson, R. E., Pitts, G. C. & Consolazio, F. C. (1944). *Amer. J. Physiol.* **141**, 575.
Ladell, W. S. S. (1947). *J. Physiol.* **106**, 237.
Ladell, W. S. S. (1948). *J. Physiol.* **107**, 465.
Ladell, W. S. S., Waterlow, J. C. & Hudson, M. F. (1944). *Lancet*, ii, 491, 527.
Lee, D. H. K., Murray, R. E., Simmonds, W. J. & Atherton, R. G. (1941). *Med. J. Aust.* **2**, 249.
Marriott, H. L. (1947). *Brit. med. J.* i, 246, 285, 328.
McCance, R. A. (1936). *Proc. Roy. Soc.* B, **119**, 245.
McCance, R. A. (1937). *Biochem. J.* **31**, 1278.
McCance, R. A. (1938). *J. Physiol.* **92**, 208.
Moss, K. N. (1922). *Proc. Roy. Soc.* B, **95**, 181.
Nadal, J. W., Pedersen, S. & Maddock, W. G. (1941). *J. clin. Invest.* **20**, 691.
Newburgh, L. H. & Johnston, M. W. (1934). *J. Nutrit.* **7**, 107.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, 1st. ed. **2**. London: Baillière, Tindall and Cox.
Phillips, R.A., Van Slyke, D. S., Dole, V. P., Emerson, K., Hamilton, P. B. & Archibald, R. M. (1942). *Copper Sulphate Method for Measuring Specific Gravity of Whole Blood and Plasma.* Washington cmr. report: Bureau of Medicine and Surgery.
Talbott, J. (1935). *Medicine*, **14**, 321.
Weiner, J. S. (1945). *J. Physiol.* **103**, 36 *P*.
Winkler, A. W., Elkinton, J. R., Hopper, J. & Hoff, H. E. (1944). *J. clin. Invest.* **23**, 103.

J. Physiol. (1949) 108, 451–457 612.27.611.975

THE EFFECT OF THE APPLICATION OF AN ARTERIAL OCCLUSION CUFF TO THE WRIST ON THE BLOOD FLOW IN THE HUMAN FOREARM

By D. McK. KERSLAKE

From the Royal Air Force Institute of Aviation Medicine, Farnborough

(*Received* 29 *July* 1948)

The venous occlusion plethysmograph has been widely used for the measurement of forearm blood flow. It was pointed out by Grant & Pearson (1938) that the rate of increase of forearm volume occurring when the collecting pressure was applied to the arm might be affected by the venous return from the hand entering the forearm. In order to remove this source of error they applied a blood-pressure cuff to the wrist. This was inflated to a high pressure (about 200 mm. Hg) and thus the hand was almost completely cut off from the general circulation. The application of this pressure squeezes some blood into the forearm, and it was observed that after a sudden initial increase the forearm volume diminished, becoming constant after about 10–15 sec. It was assumed that by this time the forearm blood flow, if affected by this procedure, would have settled down to a steady rate. It was the practice of these workers to record three inflow curves in quick succession, starting the first 15 sec. after the application of the wrist cuff. The average of these three was taken as the forearm blood flow at the time concerned. It was observed that the flows measured in this way were the same as those obtained by inflating the wrist cuff and leaving it inflated for half an hour or so, taking groups of three readings meanwhile at the same time intervals as before. For this reason the inflation of the wrist cuff just prior to each series of inflow curves was regarded as justifiable. The relation between the three consecutive readings following the application was not described.

It was felt that a fuller examination of the possible changes in blood flow resulting from the inflation of the wrist cuff would be desirable. The results of such an investigation are described in this paper.

METHODS

Since records of forearm blood flow were required at frequent and regular intervals, the automatic blood-flow recorder described by Kerslake (1949) was used for these experiments. The same system of volume recording was used since it presents no disadvantages when used at ground level,

although originally designed for use at high altitudes. The collecting cuff was a 2½ in. sphygmomanometer cuff, which completely encircled the arm. The wrist cuff was made from 1½ in. bicycle inner tubing. It completely encircled the wrist and was held in place by a band of stout rubber sheet. The water-bath temperature was 34° C.

The subjects were healthy men aged 18–34. Of the eight subjects used four had previous experience as subjects of blood-flow experiments and four had not had such experience. The subject was seated at rest in a room at about 22° C. while the plethysmograph and cuffs were being fitted. As soon as arrangements for the experiment were complete the collecting pressure cycle was started. This consisted of alternate periods of application and release of the collecting pressure, each lasting 5 sec., so that one inflow curve could be recorded every 10 sec. Preliminary experiments had shown that this procedure produced no detectable change in the forearm blood flow, but in order to ensure that its influence should not affect the results of the current investigation the cycle was continued throughout the whole experimental session without interruption.

After about 5 min. the wrist cuff was rapidly inflated from a reservoir containing air at a pressure of 250 mm. Hg. This inflation, resulting in a wrist-cuff pressure of 230–240 mm. Hg coincided with the end of one of the periods of application of the collecting pressure. The wrist cuff remained inflated during the next ten periods of venous occlusion (100 sec. in all) and was then released. The inflation of the wrist cuff in this way was repeated at intervals of 5 min. On the first two or three occasions no records of forearm flow were taken, so that the subject had been exposed to the conditions of the experiment for about 15 min. before observations were begun. Thereafter records were taken of the changes in forearm volume during the ten periods of venous occlusion immediately following each inflation of the wrist cuff. Such a series of observations will be referred to as a 'run'. Usually twelve runs were recorded in each experiment. Any runs which were marred by movement of the subject during the recording were rejected; otherwise in the absence of such imperfections the first two runs were neglected, so that ten sets of results were used in the subsequent analysis.

In some of the experiments arrangements were made for the wrist cuff to be inflated from the same source as the collecting cuff, as described by Barcroft & Edholm (1945). In these cases this procedure was used throughout except during the periods of high wrist-cuff pressure, which were arranged as before. Records were taken during the four periods of venous occlusion immediately preceding the application of the high pressure to the wrist cuff, and during the ten subsequent periods as before.

In a few experiments a second wrist cuff was applied to the right wrist, the left forearm being in the plethysmograph. In these cases the runs were divided into two groups. In the control group the left wrist cuff was inflated and records of blood flow taken during the next sixteen periods of venous occlusion. These results furnished some evidence concerning blood-flow changes during this additional minute. In the other group the procedure was the same except that the cuff on the right wrist was inflated from a high-pressure reservoir at the end of the tenth reading, remaining inflated during the next six. Both wrist cuffs were then released.

Expression and statistical treatment of results. When a series of runs obtained on one subject at one sitting was plotted there seemed to be no consistent pattern of response, and it was realized that any changes due to the inflation of the wrist cuff must be comparable with or smaller than the spontaneous fluctuations of forearm flow arising from sources which could not be controlled. When ten runs were averaged it was found that a fluctuation of the order of 0·8 c.c./100 c.c. forearm/min. occurred during the first minute, the last five readings showing little variation (Fig. 1).

The general level of blood flow during different runs was not constant, the scatter being much greater than the extent of the changes seen in the curve of averages. If standard deviations were determined for each time interval after the application of the wrist cuff it was clear that they would include not only the variance of the response to inflation, but also the very large variance of the general flow level during the different runs. A response which was quite constant on different occasions might therefore be entirely masked by this irrelevant factor unless steps were taken to eliminate it. From the curve of averages it seemed that the blood flow became nearly constant after

the first minute, and it was decided to use the average of the seventh to tenth readings in each run as a common base-line to which the different runs could be reduced. In this way the differences in general blood-flow level would be eliminated from the calculation of the variance of the response, and the significance of the fluctuation present in the curve of averages could be determined.

Each reading of each run was thus expressed as a difference from the mean of the seventh to tenth readings in that run. The ten results obtained in this way for each time interval (from the results of ten runs) were averaged, and the variance of the observations at each time interval calculated. This method of calculation does not allow for changes in general blood-flow level during the period of each run, but these changes were small, since the general levels in consecutive runs did not differ very greatly. However, the standard deviation of the results might be expected for this reason to decrease slightly from the first reading onwards as far as the seventh. Sufficient data were not obtained for the calculation of the extent of this expected change, and so the figures for standard deviation can only be considered in relation to the confidence limits they supply for the time intervals concerned. An accurate estimate of the true scatter of the results at each time interval cannot be made.

RESULTS

The curve of averages obtained in this way was similar in shape to the crude average curve in Fig. 1, but the confidence limits of each point could now be determined. On the basis of ten results the fluctuation just reaches a significant level when the points are considered individually. A full calculation of correlation would probably strengthen the significance, but is not practicable for a curve of this shape. Fig. 2 shows the average curves for eight subjects. With the exception of one, which returns to zero more rapidly than the others and then rises above the zero line, the curves for both experienced and inexperienced subjects show no significant differences from one another. It is not possible to assign precise time values to the various readings in each run, since each observation occupies 5 sec. The time scale has therefore been expressed in terms of the readings, rather than in terms of time, the significance of which would be doubtful. Each curve represents the results of ten runs.

In order to obtain more precise information about the nature of this response, the results from the seven similar subjects were pooled. Twelve additional runs on one of them have also been included. The grand average for each time interval was calculated, and the standard deviation calculated with reference to this. The confidence limits were then found. The mean curve for the seven subjects is shown in Fig. 3, where the confidence limits of mean flow and of time are indicated. Each point on the curve represents the mean of eighty-two observations.

The general levels of blood flow were 2–4 c.c./100 c.c./min. in thirty-eight runs, 4–6 c.c. in forty-one runs, and 6–8 c.c. in three runs. The general level in the runs on the subject omitted from the final average was 2–4 c.c. No correlation can be found between the general level of blood flow in the runs and the extent of the fluctuation in blood flow.

The relationship of the blood flow after the inflation of the wrist cuff to the blood flow present beforehand was investigated as described earlier, by using the technique of simultaneous inflation of the wrist and collecting cuffs to the

same pressure. The four observations taken in this way before the application of the high pressure to the wrist were compared with the last four high-pressure

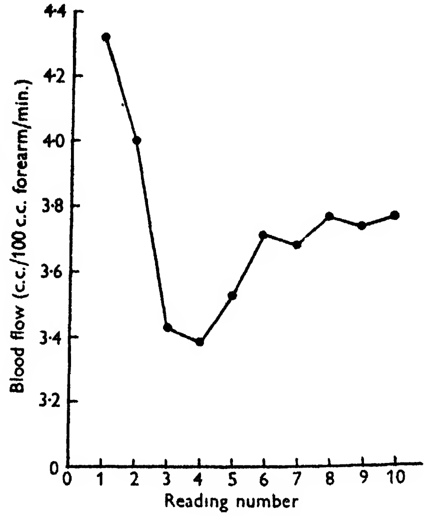


Fig. 1. Average of ten runs of subject B.H. Wrist cuff pressure applied 5 sec. before the start of the first reading. Interval between readings 10 sec.

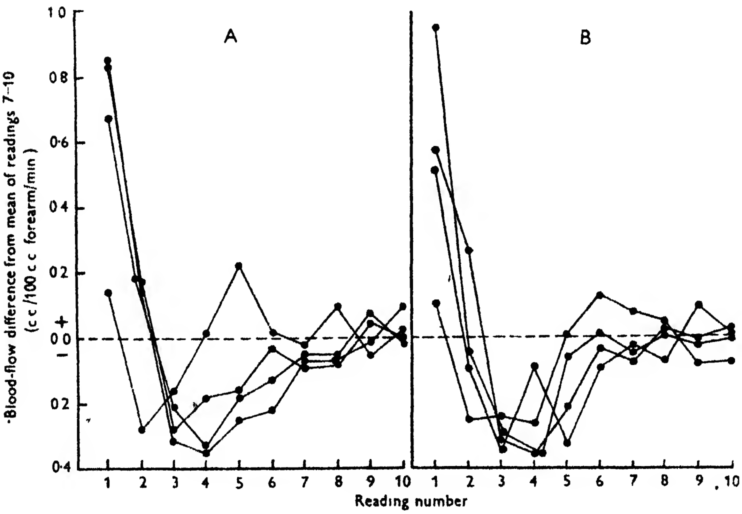


Fig. 2. Average curves for ten runs on each of eight subjects, the runs being reduced to a common base-line as described in the text. Group A inexperienced subjects. Group B experienced subjects.

readings in that run. In some cases there was evidence that blood from the hand might be coming back under the wrist cuff during the low-pressure readings. The criteria for the recognition of this factor were necessarily rather vague. If

the forearm volume record became steeper during the period of occlusion, or if the nature of the pulse beats present in it was different from those occurring in the high-pressure records, break-through from the hand was assumed to be present and the run was neglected.

Nineteen runs were observed in which no trace of break-through could be detected. The average value of the blood flow in the seventy-six low-pressure readings was 3·85 c.c./100 c.c. forearm/min. The average flow for the last four high-pressure readings in these runs was 3·82 c.c./100 c.c. forearm/min. From these results it may be supposed that the steady level of blood flow reached after the first minute following the application of the high-pressure wrist cuff does not differ appreciably from that present before the cuff is applied.

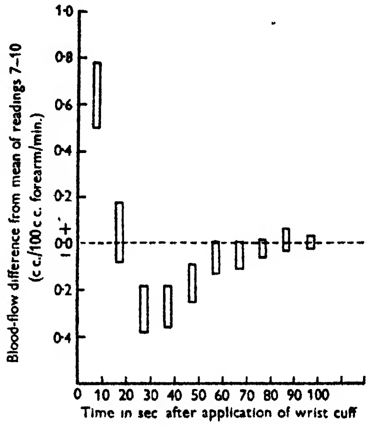


Fig. 3. Pooled results of a total of eighty-two runs on seven subjects. Rectangles represent 95% confidence limits of mean blood flows, and approximate spread of results in the time scale.

The experiments on inflation of a cuff on the right wrist, the left forearm being in the plethysmograph, led to two conclusions. In the control group it was found that the blood flow during the time from $1\frac{1}{2}$ to $2\frac{1}{2}$ min. after the application of the high-pressure cuff to the left wrist remained at the steady average level it had reached by the end of the first minute. The blood flow during the same period was not appreciably altered when the cuff on the right wrist was inflated. Only eighteen runs of this type were carried out, so that the standard errors of the results are rather large, but the findings demonstrate conclusively that if inflation of the cuff on the right wrist has any effect on the blood flow in the left forearm, it is very much smaller than the change produced by the inflation of the left wrist cuff. The results are given in Table 1.

Table 1. Mean values for forearm blood flow (relative to the average of the seventh to tenth readings) in the eleventh to sixteenth readings, with and without the inflation of a cuff on the right wrist. Results of ten runs with right wrist cuff and eight control runs

Reading number...	11	12	13	14	15	16
Right Cuff:						
Average	+0·03	0·00	−0·07	+0·02	−0·04	+0·07
S.E.	±0·09	±0·11	±0·08	±0·07	±0·07	±0·08
Control:						
Average	−0·06	+0·01	0·00	−0·03	+0·09	+0·04
S.E.	±0·07	±0·10	±0·07	±0·08	±0·11	±0·08

DISCUSSION

The results described in this paper demonstrate the existence of a regular fluctuation in the apparent arterial inflow to the forearm during the first minute following the inflation of the arterial occlusion cuff round the wrist. The steady average flow rate which is reached at the beginning of the second minute is maintained for at least 90 sec., and there is no reason to suppose that it varies thereafter. It would seem, therefore, that when estimations of forearm blood flow are required at intervals of 5 min. or more, inflation of the w[illegible] can safely be performed on each occasion, provided that an i[illegible] is allowed to elapse between this and the recording of [illegible] short se[illegible] curves. The maintenance of the wrist cuff at high pressure for lo[illegible] a source of discomfort to the subject and possibly causes changes i[illegible] flow. On the basis of these results it appears to be an unnecessary [illegible] unless it is desired to measure the blood flow at more frequent inte[illegible] those cited above.

The relation of the observed forearm blood flow to the flow which [illegible] present if measurements were not being made does not lend itself to direct investigation, since an independent method of estimation of blood flow does not exist. The results obtained by the simultaneous inflation of the wrist cuff and the collecting cuff to the same pressure are, however, substantially the same a[illegible] the flow rates recorded with the high-pressure cuff after time has b[illegible] allow[illegible] for the blood flow to reach a steady level. While the interpret[illegible]on of the low-pressure readings is open to question, for reasons outlined above, the close similarity of these results suggests that the high-pressure cuff alone does not produce any permanent change in forearm blood flow. The possible effect of the application of the plethysmograph and collecting pressure cannot be assessed.

The cause and mechanism of the initial fluctuation in apparent forearm blood flow after the application of the high-pressure wrist cuff have not been investigated, but a few observations relevant to this question have been made. It would appear that since no change in blood flow occurs when a cuff is inflated round the opposite wrist, the effect must be a local one.

If the circulation to the forearm is arrested by the application of a high-pressure cuff round the arm, inflation of the wrist cuff produces a sustained increase in forearm volume, presumably by squeezing blood into it, and possibly by some displacement of the limb or the end diaphragm of the plethysmograph. This increase in forearm volume, real or apparent, is complete within half a second. The high flow rate observed during the first reading after the application of the high-pressure wrist cuff cannot therefore be due to the slow extrusion of blood and tissue fluid from beneath the wrist cuff into the forearm.

If the wrist cuff is inflated without obstruction of the arm a similar sudden rise in forearm volume is recorded, and this at once begins to fall, reaching a steady level after about 15 sec. This is in agreement with the observations of Grant & Pearson (1938). If the sudden introduction of extra blood from beneath the wrist cuff produced severe congestion in the veins of the forearm, raising their pressure to a level comparable with the collecting pressure, the first reading might be lower than the arterial inflow present at that time. As it was, in fact, greater than the subsequent and previous recorded inflow rates, this possibility cannot explain the observed change. Moreover, the congestion of 100 c.c. forearm disappeared almost completely by the time the third and may be sulings, which show a reduced inflow rate, are taken.
blood flowen stoppage of the considerable total blood flow to the hand might following th to result in alterations in the haemodynamics of the arterial wrist n in the forearm. The kinetic energy of this blood presumably becomes that erted into potential energy in the form of a local increase in arterial essure, and it may be possible to account on this basis for the early increase in blood flow which has been observed. Apart from this, reflex or humoral mechanisms may be concerned. It is important, however, to bear in mind that these observations can only be regarded as measurements of the apparent forearm blood flow, and it is possible that the fluctuation described does not accurately represent the actual changes in arterial inflow.

SUMMARY

1. Observations were made of the changes in forearm blood flow occurring immediately after the application of an arterial occlusion cuff to the wrists of eight subjects.

2. Responses of different subjects were essentially similar, consisting of an initial increase in blood flow followed by a decrease, the flow rate becoming stable at an intermediate level after approximately 1 min.

3. This stable level of blood flow did not differ significantly from the flow rate determined by simultaneous inflation of the wrist cuff and collecting cuff to the collecting pressure immediately before the inflation of the wrist cuff to high pressure.

4. The inflation of an arterial occlusion cuff on the right wrist produced no change in the blood flow in the left forearm.

I am grateful to the Director General of Medical Services, R.A.F., for permission to publish this paper.

REFERENCES

Barcroft, H. & Edholm, O. G. (1945). *J. Physiol.* **104**, 161.
Grant, R. T. & Pearson, R. S. E. (1938). *Clin. Sci.* **3**, 119.
Kerslake, D. McK. (1949). *J. Physiol.* **108**, 398.

J. Physiol. (1949) 108, 458–466 612.172.4:021

THE TIMING OF CERTAIN CIRCULATORY EVENTS IN MAN

By W. SCHLAPP and A. G. WALKER

From the Department of Physiology, University of Manchester

(*Received* 12 *August* 1948)

The use of electronic methods of investigation is by now well established in many fields. This paper describes an application to the timing of the Korotokow blood-pressure sounds, the heart sounds and the arterial pulse wave.

METHODS

Electrically the most striking feature of the cardiac cycle is the R-wave of the electrocardiogram. This change has been utilized immediately, or after a suitable delay, to trigger the sweep of a recording cathode-ray tube just before the phenomenon under observation is expected to take place.

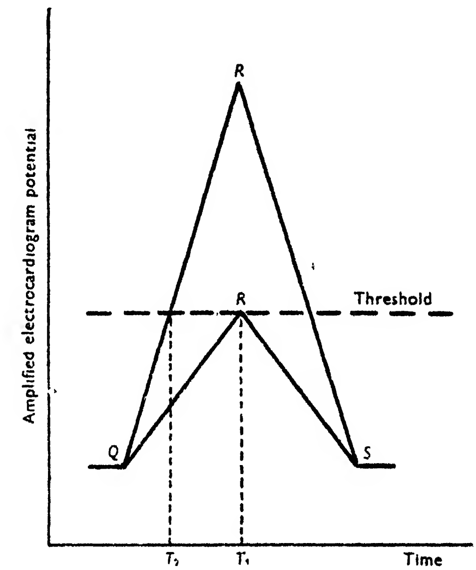


Fig. 1. Effect of magnitude of amplified R-wave on time of triggering.

An important consideration as regards timing is the precision with which the R-wave can be used for this purpose. The device employed is a threshold one; the amplified voltage of the R-wave is made to fire a gas discharge triode. The degree of amplification of the R-wave will affect the time

of initiation of the sweep in the way illustrated in Fig. 1. The over-threshold wave will trigger the device at T_2, while the threshold one will do so at T_1. To eliminate this effect the amplification was always adjusted to be just above threshold.

A rather similar error may arise from respiratory alterations in magnitude of the R-wave such as result from changes in position of the electrical axis of the heart. If the change were a great one it might operate in the same way as is illustrated in Fig. 1. We have regularly used lead II in this work. The respiratory variation in this lead may be 10% with normal breathing. The timing error can then be worked out by a geometrical construction (similar to Fig. 1) based on the duration of the QRS-wave and its magnitude; the error does not exceed 5 msec. No greater respiratory variation in the electrocardiogram has been met, but such can occur in pathological conditions and particularly in lead III. The addition of a differentiating device to the amplifier such as is described later in this paper in connexion with the timing of the pulse wave would eliminate this source of error.

Another error may arise from the presence of potentials of extraneous origin in the amplified electrocardiogram. The commonest of these is the 50-cycle mains ripple often picked up on the leads from the patient. This might in some circumstances advance or retard the timing to the extent of 0·02 sec.; it can be eliminated in the amplifier.

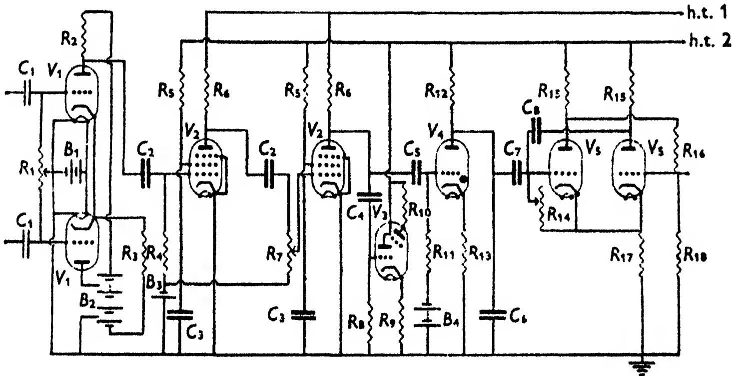


Fig. 2. Circuit diagram:

Resistances		Condensers	Valves
$R_1 = 50$ KΩ	$R_{11} = 47$ KΩ	$C_1 = 1$ mfd.	V_1 = MH 40
$R_2 = 30$ KΩ	$R_{12} = 0·5$ MΩ	$C_2 = 1$ mfd.	V_2 = SP41
$R_3 = 30$ KΩ	$R_{13} = 1$ KΩ	$C_3 = 8$ mfd.	V_3 = TV4
$R_4 = 1$ MΩ	$R_{14} = 60$ KΩ in fixed steps of 5 KΩ	$C_4 = 0·005$ mfd.	V_4 = GDT4B
$R_5 = 10$ KΩ		$C_5 = 0·5$ mfd	V_5 = MH$_4$
$R_6 = 20$ KΩ	$R_{15} = 10$ KΩ	$C_6 = 0·1$ mfd.	
$R_7 = 1$ MΩ	$R_{16} = 40$ KΩ	$C_7 = 0·005$ mfd.	
$R_8 = 0·5$ MΩ	$R_{17} = 5$ KΩ	C_8 = Variable 4, 2, 1, 0·1 mfd.	
$R_9 = 0·1$ KΩ	$R_{18} = 5$ KΩ		
$R_{10} = 1$ MΩ			

The amplifier used is a conventional one and a circuit which has been found satisfactory is given in Fig. 2. The input stage (V_1) is battery operated. The remaining two stages (V_2) are mains operated with adequate smoothing. Mains ripple picked up from the patient is controlled by the potentiometer (R_1) in the input stage which is of the discriminating type described by Tönnies (1938). The control (R_1) is used while observing the 'magic eye' indicator (V_3); mains ripple defocuses this device. The output of the amplifier, controlled by the potentiometer (R_7) (Fig. 2), fires a gas discharge triode (V_4). This in its turn actuates a delay device so that after some fixed interval the beam of the oscilloscope is triggered.

The delay device depends upon the principle of the multivibrator. By a suitable choice of condensers (C_8) and resistances (R_{14}) (Fig. 2) a wide range of delays is obtained. If a step potentiometer is used for (R_{14}) the delay can be set to a chosen value determined by calibration on an oscilloscope against a known frequency.

The timing of the Korotokow sounds

The method has been applied in timing the Korotokow blood-pressure sounds. These have been picked up by means of a crystal microphone over the brachial artery at the elbow during the deflation of a sphygmomanometer cuff on the upper arm. A resistance capacity-coupled amplifier was used. Attention was directed to the times of occurrence and not to the quality of the sounds produced.

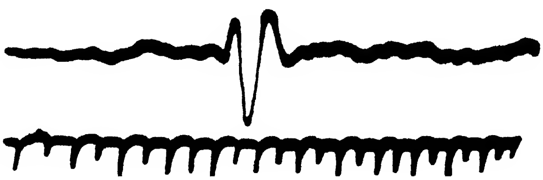


Fig. 3. Oscillograph record of Korotokow sound.

In Fig. 3 a Korotokow blood-pressure sound has been recorded by means of the technique described above. A delay of 0·12 sec. was interposed between the occurrence of the R-wave of the electrocardiogram and the triggering of the oscilloscope beam, and this time should be added to that read off from the tracing if the absolute R-wave-Korotokow sound time interval is to be measured.

By using moving bromide paper it is easy to record successive Korotokow sounds while the sphygmomanometer cuff is being deflated. A typical record of this taken from an adult appears in Fig. 4a on which the progressive fall of cuff pressure is also recorded. It will be seen that successive sounds become earlier during deflation of the cuff. This fact has been noted previously by Korns (1926), and is related to the obliquely rising front of the percussion wave of the pulse.

In the tracing (Fig. 4a) it will be seen that by joining together corresponding points in successive Korotokow sounds a curve which closely resembles the rising front of the pulse wave is obtained. The curve is, however, not quite a smooth one and deviations are often marked as may be seen in Fig. 4b, which is taken from an adolescent. It is likely that the deviations have a respiratory origin and this can be confirmed as follows.

Respiration may influence the expected time of occurrence of the Korotokow sound in two ways: both are connected with effects upon the blood pressure. A change of conduction velocity of the pulse wave will result from respiratory

changes in blood pressure. Attention was originally called to this by Hickson & McSwiney (1924) and the phenomenon will be dealt with in a later section of this paper.

The form of the pulse wave also plays a part in determining the time of occurrence of the Korotokow sound. The mechanism is illustrated in Fig. 5. *A* and *B* represent respectively pulse-wave fronts at the maximum and minimum

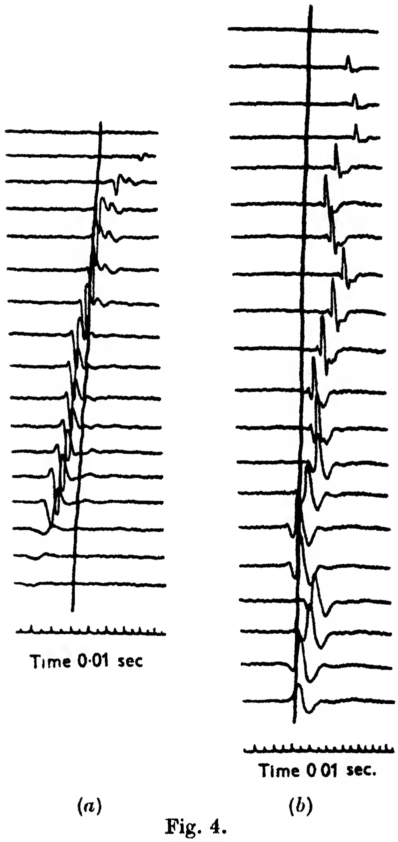


Fig. 4.

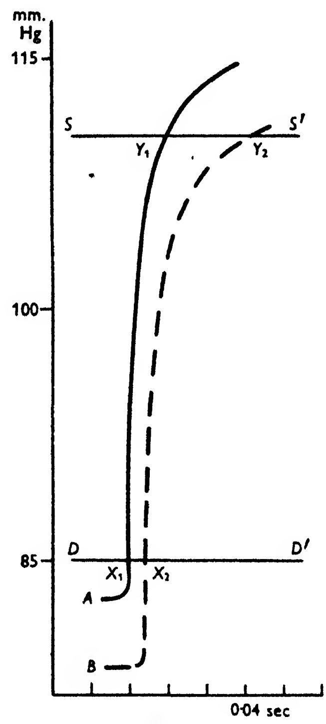


Fig. 5.

Fig. 4. Records of successive Korotokow sounds during deflation of sphygmomanometer cuff. Diagonal line records the steady fall of pressure in the cuff over a range somewhat in excess of the pulse pressure.

Fig. 5. Diagram illustrating effect of cuff pressure and respiration on time of Korotokow sound.

arterial pressures occurring during a respiratory cycle. If the pressure in the sphygmomanometer cuff be near diastolic and is represented by the line DD' the sound will occur at X_1 and X_2, and the form of the wave front will have little influence on the time of its occurrence. On the other hand, if the pressure in the sphygmomanometer cuff be near systolic as is represented by the line SS', the sounds will be heard at Y_1 and Y_2.

In Fig. 6 tracings are reproduced to bring out these points. In both there are stethograph records as well. It will be seen that when the pressure in the cuff is near systolic there is a larger respiratory variation in timing than when the pressure is at a lower level. A consideration of the amplitude (or loudness) of the sound in the record at the higher cuff pressure shows that it is least at the time of inspiration. This means that the pressure difference on the two sides of the cuff is least at this time, and it is clear that it increases with expiration. These observations on the amplitude of the sounds serve to establish the

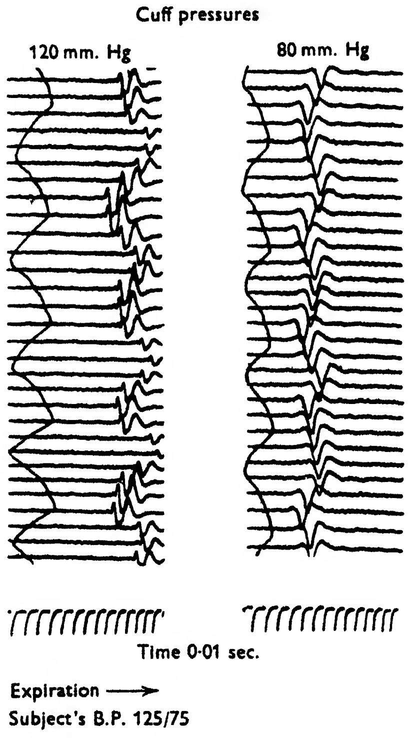


Fig. 6. Time of Korotokow sounds with different cuff pressures showing effect of respiration.

relation of the systolic blood pressure to respiration. Blood pressure is highest in this subject at expiration. At this time the velocity of conduction of the pulse wave should be maximal and the factor due to the form of the pulse wave will be least effective (cf. X_1 and Y_1 in Fig. 5). At the lower levels of blood pressure both factors co-operate to make the time of occurrence of the sounds later (cf. X_2 and Y_2 in Fig. 5).

There are not as yet sufficient observations to determine finally which subjects show the greatest respiratory effects, but it is clear already that these are generally less well marked in older subjects. This recalls similar facts in relation to sinus arrhythmia.

Timing of the heart sounds

In Fig. 7*a*,*b* successive first and second heart sounds are recorded with a crystal microphone and amplifier. The records include stethograph tracings. In Fig. 7*a* the oscilloscope beam was directly triggered by the *R*-wave of the electrocardiogram. The beginning of the first heart sound is recorded; the time

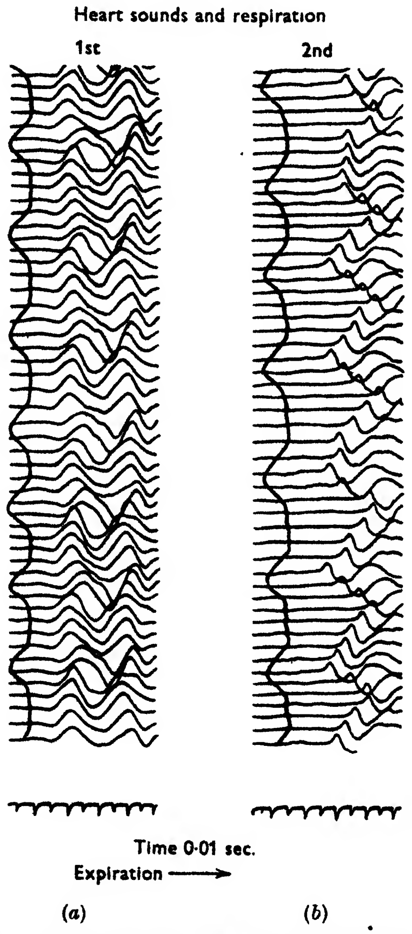


Fig. 7. Timing of first and second heart sounds.

of its occurrence is sensibly constant. Fig. 7*b* records the time of occurrence of the second heart sound on the same time-scale but with the interposition of a delay of 0·35 sec. There is a rhythmical variation in the duration of the *R*-wave-second heart sound interval and it has the respiratory frequency.

As this tracing was obtained from the subject who provided Fig. 6 it is clear that the second heart sound is later at expiration when, as we have seen, blood pressure is higher. If we regard the occurrence of the second sound as marking

the end of ventricular systole it appears that at higher pressures systole lasts longer. The absolute values of the time differences are small and do not exceed 0·03 sec. It would be interesting to discover whether the lengthening of systole occurs in both the isometric and ejection phases, but the methods used here do not lend themselves to the solution of this problem.

Timing of the pulse wave

Hickson & McSwiney (1924) originally drew attention to the respiratory variation in the velocity of propagation of the pulse wave. The method described here makes continuous recording possible.

The time of arrival of the pulse wave at a point has been determined by means of a carbon granule recorder which is represented diagrammatically in Fig. 8. Similar devices have been used previously, as, for example, by Staehelin & Muller (1932). As in the case of a carbon microphone compression reduces the resistance and the potential changes thus produced by the pulse wave are recorded on the oscilloscope after suitable amplification.

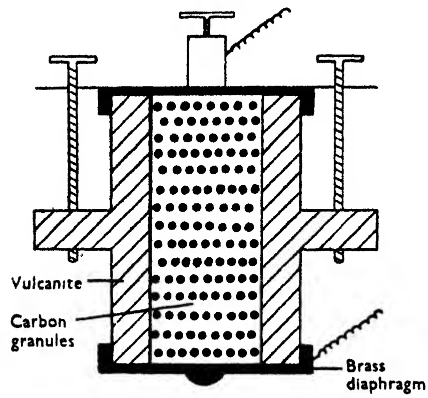


Fig. 8. Diagram of carbon granule recorder.

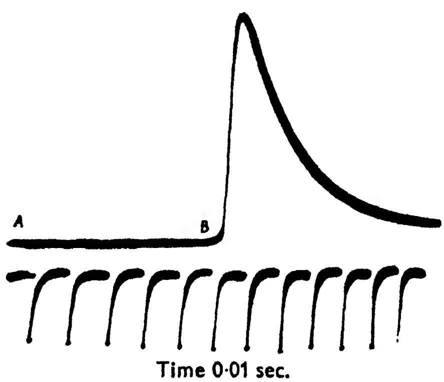


Fig. 9. Differentiated pulse wave recorded at the wrist showing only the first part.

As we have not been concerned with the form of the pulse wave, but only with the time of its occurrence, we have employed a 'differentiating' action in our amplifier. This is done by using a short time-constant coupling between two of the stages. A record of the first part of the radial pulse made in this way is seen in Fig. 9. In a 'differentiated' record only the changes of gradient are recorded and the value lies in the precision with which the commencement of the percussion wave can be determined. It is obvious that measurement of the distance *A–B* (Fig. 9) taken together with the known delay will determine the time of arrival of the pulse wave.

In making continuous records of changes in the *R*-wave-radial pulse time it is convenient to cover the face of the oscilloscope except for a narrow slit in the

direction of the time axis. Successive traverses of the beam are thereby recorded as lines which terminate when the beam is deflected. In fact the distance *A–B* (Fig. 9) can repeatedly be recorded by this means on moving bromide paper.

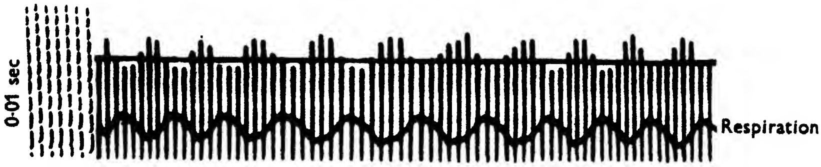


Fig. 10. *QRS*-radial pulse time. Inspiration downwards. Delay 0·17 sec.

In Fig. 10 such a record is shown. The oscilloscope beam is triggered by the *R*-wave of the electrocardiogram after a delay of 0·17 sec. and the record is taken from the radial artery. It is evident that the time of arrival of the pulse wave at the wrist varies with respiration for the relation to the stethograph tracing is clear. At expiration when the pressure is highest the pulse wave arrives earlier.

It may be doubted whether the changes in timing demonstrated—amounting in this case to 0·02–0·03 sec.—can be accounted for entirely by changes in pulse-wave velocity. The time interval measured includes the isometric phase of ventricular contraction, and any interval which elapses between the occurrence of the *R*-wave of the electrocardiogram and the commencement of isometric contraction. It is unlikely that the latter interval is subject to respiratory variation having regard to the constancy of timing of the first heart sound already demonstrated. The isometric phase may be subject to respiratory variation, but the amount must be small as its duration is only some 0·04 sec. The greatest part of the time difference must therefore be attributed to changes in propagation rate of the pulse wave.

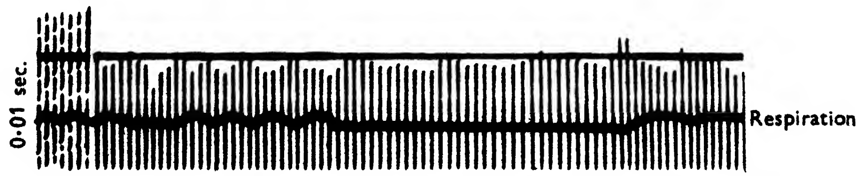


Fig. 11. Brachio-radial pulse time. Inspiration downwards. Brachio-radial distance 27 cm. Delay nil. Age 47. B.P. 135/90.

It is known that the structure of an artery influences the propagation rate through its elastic properties. In general, propagation is slower in the larger arteries (Dow & Hamilton, 1939). Changes in propagation rate can also be shown to occur in relatively short and uniform arteries such as the radial. In Fig. 11 the beam of the oscilloscope was triggered directly by the arrival of the pulse wave in the brachial artery at the elbow; a carbon granule recorder was

used. A second recorder at the wrist marked the time of arrival of the pulse wave by deflecting the beam. A stethograph record is included. The particulars regarding the subject are given on the figure and the conduction rate over the radial artery ranges from 5 to 7 m./sec. The respiratory variation is well brought out on the tracing. It should be noted that rhythmical variations in rate of pulse-wave propagation occur during a period of voluntary apnoea. It is likely that these are dependent on blood-pressure changes.

SUMMARY

1. A method of timing circulatory events which depends on triggering the beam of a cathode-ray tube is described. Examples of its use with the *R*-wave of the electrocardiogram are given, and the errors which may arise are discussed.

2. The timing of the Korotokow blood-pressure sounds shows that there is a respiratory variation due partly to the shape of the wave and partly to differences in its conduction velocity; the variation is related to respiratory changes in blood pressure.

3. The heart sounds have been timed. It is shown that the interval between the *R*-wave and the first sound is constant, while that between the *R*-wave and the second sound is subject to respiratory variation. The observations are in accord with the view that systole is of longer duration when the blood pressure is raised.

4. Continuous records of changes in pulse-wave velocity have been made. The observation of Hickson & McSwiney that the velocity is affected by respiration is confirmed and it is shown that spontaneous changes in pulse-wave velocity occur during voluntary apnoea.

Grateful acknowledgement is made to the Medical Research Council for a grant for apparatus to one of us (W. S.).

REFERENCES

Dow, P. & Hamilton, W. F. (1939). *Amer. J. Physiol.* **125**, 60.
Hickson, S. & McSwiney, B. A. (1924). *J. Physiol.* **59**, 217.
Korns, H. M. (1926). *Amer. J. Physiol.* **76**, 247.
Staehelin, R. & Muller, A. (1932). *Z. exp. Med.* **83**, 347.
Tönnies, J. F. (1938). *Rev. Sci. Inst.* **9**, 95.

J. Physiol. (1949) 108, 467–481 577.174.5.612.129

THE ESTIMATION OF ADRENALINE AND ALLIED SUBSTANCES IN BLOOD

By J. H. GADDUM, W. S. PEART and M. VOGT

From the Department of Pharmacology, University of Edinburgh

(*Received* 10 *September* 1948)

Much work has been devoted to the search for a really sensitive and specific method of detecting and estimating adrenaline and allied substances in blood. This search has gained importance in recent years because of the desire to identify the substances liberated by adrenergic nerves. These substances can only be expected to appear in very low concentrations, and sensitive and specific tests are needed for their study. This paper describes part of the search for such tests and is a continuation of the work of West (1947*a*, *b*) in this department.

The most sensitive tests for adrenaline are pharmacological tests, but no single test is really specific by itself. A number of other sympathomimetic amines are known to have effects like those of adrenaline, but they can sometimes be distinguished by the method of parallel quantitative assays. If the adrenaline-equivalent of a solution is estimated quantitatively by several different methods, and the results differ significantly among themselves, adrenaline cannot be the only active substance in the solution. If the results agree among themselves, then the evidence supports the theory that the solution contains adrenaline, but its value depends on the use of pharmacological methods which vary independently in their sensitivity to drugs closely allied to adrenaline. If small changes in the molecule affect all the tests equally, then parallel quantitative assays are of little value. One of the objects of the present investigation was to discover a set of tests which would vary independently in their response to sympathomimetic amines, so that they could be used to distinguish these amines from one another.

One of the main difficulties in the estimation of adrenaline in blood is due to the fact that when blood is removed from the body various other pharmacologically active substances are formed in it or released from the cells (Gaddum, 1936; Reid & Bick, 1942; Zucker, 1944). Two of these interfering substances are histamine and an adenosine compound (Barsoum & Gaddum, 1935), but there is at least one other active substance which has not yet been identified.

Acetylcholine can be estimated in blood by using pharmacological tests which are specific for choline esters, but the known tests for adrenaline and allied substances are not specific, and can only yield useful information if measures are taken either to prevent the liberation of the interfering substances, or to remove them from the plasma afterwards. These two possibilities have been explored and the results are discussed below. The presence of these active substances complicates most pharmacological experiments with blood and the techniques used in the study of blood-adrenaline may have applications in the study of other substances.

METHODS

Dilute solutions of adrenaline and allied drugs are apt to be unstable. Drugs have therefore been dissolved in 0·9% NaCl containing ascorbic acid (10^{-5}). Such solutions are stable for many hours.

Doses are generally given in terms of mμg. of the base (1 mμg. = 10^{-6} mg.). In comparing different drugs it would have been better to use equimolar solutions, but this would not have made much difference, since the molecular weights of most of the substances studied are nearly equal.

TABLE 1. Drugs used

		$3,4(HO)_2C_6H_3C$	—CH	—N
Hydroxytyramine HCl	Light and Co.	H_2	H	H_2
Noradrenaline HCl (L and DL)	Sterling Winthrop	H.OH	H	H_2
L-Adrenaline	B.D.H.	H.OH	H	$H.CH_3$
Epinine HCl	Burroughs Wellcome	H_2	H	$H.CH_3$
Adrenalone	Light and Co.	O	H	$H.CH_3$
DL-corbasil HCl	Sterling Winthrop	H.OH	CH_3	H_2
DL, α-ethylnor-adrenaline HCl	Sterling Winthrop	H.OH	C_2H_5	H_2
DL, *N*-ethylnor-adrenaline HCl	Sterling Winthrop	H.OH	H	$H.C_2H_5$
DL, *N*-isopropylnor-adrenaline HCl	Sterling Winthrop	H.OH	H	$H.CH(CH_3)_2$
DL, *N*-methyladrenaline	I.G. Farben	H.OH	H	$(CH_3)_2$

Tyramine HCl, ephedrine HCl, histamine acid phosphate, ergotoxine methanesulphonate (B.D.H.), neoantergan (pyranisamine maleate, May and Barker), dihydroergotamine methane-sulphonate (Sandoz).

Interfering substances. In experiments on interfering substances heparinized blood from cats and man was centrifuged at 2000–3000 r.p.m. for 10 min. and various fractions were removed with teat pipettes. The plasma still contained platelets, which were separated in some experiments by centrifuging at 4500 r.p.m. for 20 min. The other fractions contained mostly erythrocytes, or mostly leucocytes. Each was mixed with an equal volume of distilled water to lyse the cells and then used in pharmacological tests. Some of these fluids were dialysed for 4 hr. in apparatus like that described by Verney (1926), but made of perspex, using cellophane 400S of the British Cellophane Company. The receptor fluid was either distilled water, 0·005% ascorbic acid, 1% glycine in N/100-HCl or N/100-HCl. The dialysates were adjusted to various pH values with N/100-HCl and shaken with alumina of the kind used by Shaw (1938). This adsorbs adrenaline at pH 8·5, but not at pH 4.

The collection of plasma. The simplest method of avoiding complications due to interfering substances is to prevent them from being liberated. Adrenaline and noradrenaline were found to be stable for several days in plasma at 4° C. The presence of platelets in the plasma did not appear to affect the stability of adrenaline or to lead to the appearance of undesirable quantities of inter-

fering substances. Blood was therefore centrifuged at 3000 r.p.m. for 5 min. as soon as possible, and the following precautions were taken to prevent the liberation of interfering substances before the plasma could be separated:

(1) Heparin was injected intravenously into the cat in sufficient doses to prevent clotting (1000 units/kg.). Later, after various other precautions had been introduced, it was found beneficial also to add heparin (10 units/c.c. of blood) to the tubes in which the blood was collected. This seemed to be particularly important when the plasma had to be tested on the rat's uterus and colon.

(2) The blood was collected in centrifuge tubes standing in ice, and ice was also used in the centrifuge. After separation the plasma was kept at 0–4° C. and warmed up just before testing.

(3) Glass connexions and the centrifuge tubes were treated with silicone (General Electrical Co., Drifilm 9987) to render their surfaces water-repellent.

These precautions were largely successful, but some experiments were nevertheless complicated by the release of interfering substances.

It is known that when adrenaline is added to blood it is taken up by the cells, and when the concentration is low a large proportion of it may disappear from the plasma in this way (Bain, Gaunt & Suffolk, 1937), but the process is a slow one and would not be expected to affect the results of experiments in which the adrenaline is only in contact with the cells for a few minutes. A control experiment showed that this was so. Adrenaline (10^{-7}) was quantitatively recovered from the plasma of cats' blood when centrifuged after 5 min. contact with the cells.

ASSAYS

(1) *Isolated plain muscle*

de Jalon's method. Some of our best results have been obtained by a procedure recommended by de Jalon, Bayo & de Jalon (1945). Observing that the rat's uterus is very sensitive to adrenaline, but liable to show excessive spontaneous activity, these workers reduced this organ to quiescence by lowering the temperature and the calcium-content of the Locke solution in which it was immersed. They then produced contractions with acetylcholine and inhibited those contractions with small amounts of adrenaline added to the bath about 1 min. before the acetylcholine. We have used their method, except that the muscle was in a smaller bath (2 c.c.), and we have also applied the same technique to other tissues.

In our experiments on the rat's uterus the composition of the Ringer solution was as follows (g./l.): NaCl, 9, KCl 0·42, $CaCl_2$ 0·06, $NaHCO_3$ 0·5, glucose 0·5. A higher Ca/K ratio produced greater sensitivity to acetylcholine, but the maximum inhibitory effect of adrenaline was obtained with a low ratio. The temperature was 29–30° C. Large uteri appeared to be more sensitive than small ones, but pregnant or recently pregnant uteri were discarded since they did not become quiescent. Acetylcholine (0·5–1 μg.) was added to the bath every 2 min. and washed out when the effect had reached its maximum (after 30–40 sec.). The doses of adrenaline, dissolved in not more than 0·2 c.c., were added to the bath exactly 1 min. before one of the doses of acetylcholine. Another dose of adrenaline can be given as soon as the response to acetylcholine is normal. Instead of stimulating the uterus by acetylcholine, contractions may be produced by KCl in doses of 1–2 mg. This is useful if tests have to be carried out on plasma which contains atropine. Some preparations, however, lose their sensitivity to KCl after repeated administration.

A similar technique has been applied to the first 3 cm. of the rat's ascending colon which is easily recognized by the diagonal striations on its surface. This tissue was less easily reduced to quiescence than the uterus and was often used with less calcium and at a lower temperature. The solution was either the same as for the uterus or contained (g./l.): NaCl 9, KCl 0·4, $CaCl_2$ 0·03, $NaHCO_3$ 0·15, glucose 1. The temperature was 25±2° C. and was adjusted in each experiment so as to reduce the spontaneous activity to reasonable dimensions. The dose of acetylcholine was lower (0·001–0·01 μg.) than with the uterus.

In experiments with other tissues the conditions were altered according to the spontaneous activity. The rabbit's ileum usually remained spontaneously active at 20° with low calcium

concentrations and seems to be unsuitable for this technique. The guinea-pig's uterus or colon was used in low $CaCl_2$ concentrations (0·05 g./l.) at 37°. The other tissues showed little spontaneous activity and were used in normal Locke solution at 37° C. In experiments with the bladder the whole organ except the trigone was used.

Direct excitor effects. Various pieces of plain muscle were suspended in Locke solution at 37° C. In the case of the splenic capsule suitable strips were cut from the organ. Frogs' hearts were studied by Straub's method.

(2) *Perfusion with salt solutions*

Rabbit's ear. The ear was perfused with Locke solution at room temperature as described by Gaddum & Kwiatkowski (1938) with small modifications. The rabbit was first killed by a blow on the head. This was followed by low decapitation. A cannula was then tied in the right common carotid artery and perfusion started as quickly as possible at a pressure of 10–20 cm. of water; the external jugular vein was freed to allow the fluid to escape. When the blood had been washed away the branches of the artery, except that to the ear, were tied, and a cannula was inserted in the external jugular vein and connected to the outflow recorder. Injections were made in small volumes (0·05–0·2 c.c.) using the special cannula described by Gaddum & Kwiatkowski (1938).

Other tissues. Similar methods were used to perfuse various other tissues. Whole frogs were perfused from the left arch of the aorta to the posterior vena cava, and frogs' hind limbs were perfused from the abdominal aorta to the abdominal vein. These preparations were left for 4 hr. to gain their maximum sensitivity before use. Various other tissues were perfused (Table 2) at room temperature, using Locke solution for mammalian tissues and Clark solution for frogs' tissues.

In perfusing the hind limbs of rats it was found important to wash out the blood quickly because clotting is rapid. The rat was killed by a blow on the head and the aorta cannulated just above the diaphragm. Locke solution was perfused at high pressure (60–70 cm. of water) for 5 min. and then at a lower pressure (10–20 cm. of water). A cannula was then inserted in the inferior vena cava. The preparation behaved consistently for at least 24 hr., but it was often necessary to increase the perfusion pressure temporarily after doses of adrenaline to restore the flow to its initial value. The perfusion of the hind legs of mice gave similar results.

(3) *Perfusion* in situ

Various organs were perfused *in situ* in heparinized cats by a method comparable with that of Richards & Plant (1915). The blood flowed continuously from one of the larger arteries to the apparatus which pumped it into a smaller artery and so returned it to the cat. The inflow pressure was recorded and various drugs and samples of plasma were injected through the rubber tubing near the inflow cannula. The cats were anaesthetized with ether, pentobarbitone or chloralose. The details of the technique underwent various changes, but the apparatus in the later experiments is shown in Fig. 1. In some cases a small piston pump was used and in others a Dale-Schuster pump. The main disadvantage of the latter pump was that it was not easy to adjust the stroke to a small enough value. This stroke was transmitted by water to the base of a glass tube T. The blood entered and left this tube through two holes in the rubber bung B, and was separated from the water by a thin rubber membrane which covered the inner surface of the bung.

In order to facilitate the removal of air the blood left this part of the apparatus by the upper hole. The valves used were Guy Ross valves and were obtained from manufacturers of dental equipment. On emerging from the second valve the blood passed through an air space so that the flow was visible and then flowed past a filter F and a thermometer to the inflow cannula. The pressure was transmitted to a mercury manometer by means of saline.

Some trouble was experienced owing to the formation of thrombi in the circuit, and the following precautions were therefore taken. The cat received a fairly large dose of heparin (1000 units/kg.), the glass parts of the apparatus were made unwettable with silicone and a filter was included. This consisted of a small ring of perspex across which a piece of nylon gauze was tied. Thrombi did eventually form on this filter but did not interrupt experiments lasting 4 hr.

In some of the experiments an open circuit was used in which the blood flowed from the cat into an open reservoir at a rate which was controlled by a screw clip, which needed constant attention. With this open circuit changes of pressure were not transmitted through the apparatus, but no trouble was experienced from this cause even with the closed circuit, provided the perfusion pressure was kept higher than the carotid blood pressure of the cat. In some cases a second cat was used to provide extra blood, but this was not always necessary. An electric lamp was placed near the apparatus to keep it warm and the temperature recorded by the thermometer was usually 32–36°

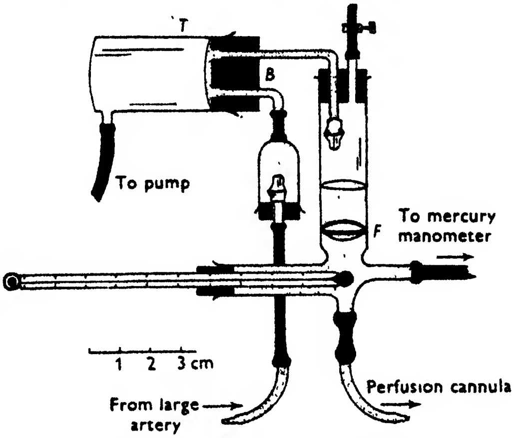


Fig. 1. Pump for perfusion *in situ.*

(4) *Nictitating membrane and lower eyelid of cat*

The technique was like that used by others (Cannon & Rosenblueth, 1937; Isola & Bacq, 1946) except that injections were made into the carotid artery instead of intravenously. The anaesthetic was allobarbitone (Dial compound Ciba) or pentobarbitone (Nembutal, Abbott) or chloralose (B.D.H.). The membrane, or lid, was sensitized by aseptic removal of the superior cervical ganglion under ether 1–2 weeks before the main experiment, and by the intravenous injection of cocaine HCl (8 mg./kg.). The contractions were recorded isotonically. To facilitate the injections a special cannula was introduced in the carotid artery. The two short horizontal arms of this cannula were so shaped that they could be tied directly into the central and peripheral ends of the artery. A third vertical arm was expanded to form a small reservoir containing air and closed by a rubber cap through which injections could be made. Heparin was injected intravenously as an anti-coagulant.

RESULTS

The results recorded in Table 2 summarize our observations on the sensitivity of various tests for adrenaline and noradrenaline. The figures given in column 3 represent the lowest dose of adrenaline likely to cause a definite effect in an average good test. These figures are liable to rather wide variations. For an accurate assay it is necessary to repeat this dose several times, and slightly larger doses would generally be more satisfactory. Special attention was paid to the effects of noradrenaline because it had become likely that this substance was liberated by certain nerves. Column 4 gives the estimates of the ratio of a dose of L-noradrenaline to the equivalent dose of L-adrenaline. In some cases these ratios were determined directly, and in others noradrenaline was used in

the racemic form and the observed ratio halved. This is thought to be justified by the fact that in a number of experiments of various kinds L-noradrenaline has always appeared to be approximately twice as active as racemic noradrenaline. This confirms previous evidence that D-noradrenaline has comparatively little pharmacological activity (Luduena, Ananenko, Siegmund & Miller, 1949). The ratios for the colorimetric and fluorescence tests are not adjusted in this way.

TABLE 2

	Test	Dose adrenaline (mμg.)	Dose L-noradrenaline / Dose L-adrenaline
	Isolated muscle in 2 c.c. bath		
(*a*) Inhibitor effects (de Jalon's method):			
Rat	Uterus	0·5	75–300
	Colon (or ileum)	15	0·2–1
Rabbit	Bladder	10	1
Guinea-pig	Pregnant uterus	20	40
	Colon	200	2
Rabbit	Ileum	200	3
	Colon	2,000	0·5
Rat	Bladder	20,000	2
(*b*) Excitor effects:			
Guinea-pig	Virgin uterus	3	2·5
Frog	Heart (Straub)	10	10–20
Rabbit	Splenic capsule	200	2·5
Rat	Vagina	200	—
	Splenic capsule	20,000	1
	Seminal vesicle	20,000	—
	Perfusion with salt solutions		
Rabbit	Ear	2	1–3
Frog	Whole animal	50	2·5
Rat (or mouse)	Hind limbs	50	2·5
Rabbit	Kidney	100	2·5
	Hind limbs	500	2·5
Frog	Lung	10,000	*c.* 1
	Other tests		
Cat	Spleen perfused *in situ*	30	0·5–1
	Nictitating membrane	50	1
Fluorescence		20	50
Colorimetric (Shaw)		40	16

Uterus. It is well known that the uterus may be excited or inhibited by adrenaline. The effect in cats is excitor in pregnancy and inhibitor in virgins or after ergot alkaloids. The effect of noradrenaline is usually in the same direction as that of adrenaline. When this effect is excitor, noradrenaline may be slightly more active than adrenaline, but when the effect is inhibitor it is much less active.

This failure to inhibit the uterus except in comparatively high concentrations is a characteristic feature of noradrenaline. This fact was discovered by Barger & Dale (1910) in experiments on cats, but West (1947*b*) found that the ratio of a dose of noradrenaline to the equivalent dose of adrenaline was particularly high in the rat's uterus, and we have also found a high ratio in the guinea-pig's uterus. In rabbits and pregnant cats these drugs stimulate the uterus and are about equally active.

The test on the rat's uterus is the most sensitive and specific of the known tests for adrenaline. It is the only one of the sensitive tests listed in Table 3 in which noradrenaline is comparatively inactive. Fig. 2 shows that in a good preparation the inhibitory effects of 1, 1·5 and 2 mμg. of adrenaline can be distinguished from one another.

In the experiment shown in Fig. 3 the sizes of successive responses were measured and plotted. In this experiment the ratio of the dose of noradrenaline to that of adrenaline was 200. In twelve experiments the ratio varied between 75 and 300 with a median value about 150.

Fig. 2.

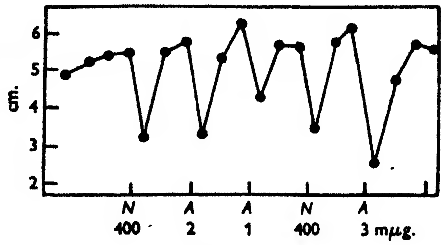


Fig. 3.

Fig. 2. Rat uterus in 2 c.c. bath. Responses to acetylcholine (1 μg. for 30 sec. every 2 min.). Inhibitory effects of adrenaline. Doses in mμg.

Fig. 3. Rat uterus in 2 c.c. bath. Inhibitory effects of adrenaline (A) and noradrenaline (N) on measured responses to 1 μg. of acetylcholine for 30 sec. every 2 min.

The effects of various other substances are shown in Table 3 and discussed later. The fact that histamine inhibits this tissue is well known. Its activity is not great, but it must be borne in mind as a possible complication of estimates of the adrenaline-concentration when this is low. Adenosine only showed an excitor effect. This is fortunate, since on other preparations the large amounts of adenosine compounds present in some tissues cause effects like those of adrenaline.

Dialysates of haemolysed cat's blood caused a slow contraction of the uterus. When all the precautions described above were taken it was generally possible to obtain control samples of plasma which had no effect on the uterus, but in some experiments, and particularly when only some of the precautions were taken, this was not achieved. In some experiments in which splenic blood was collected before and after stimulation of the splenic nerves, it was found that although the control sample might stimulate the uterus and increase the response to acetylcholine, the sample collected after stimulation of the nerves might have an inhibitory effect. In such cases it was sometimes possible to obtain an estimate of the adrenaline equivalent of the stimulus sample by adding different amounts of adrenaline to the control sample, and comparing the effects of such mixtures with those of the stimulus sample. Similar comparisons have been made with noradrenaline.

Intestine. Cat's intestine was used by Cannon & de la Paz (1911) to detect adrenaline in a conscious cat under the influence of emotion. They collected the blood from the inferior vena cava through a catheter in the femoral vein. Hoskins (1911) found that rabbit's intestine was more sensitive, and this tissue has been much used to detect adrenaline. Used in the conventional way it was not sensitive enough for our purpose. It is not very suitable for de Jalon's technique, since its spontaneous activity is difficult to suppress, but the rat's colon gives good results by this method for concentrations of adrenaline similar to those used by Hoskins. Small doses of adrenaline (such as 10 mμg.) caused a diminution of only one of the responses to acetylcholine. With larger doses the effect lasted longer and several responses were affected.

This test on the rat's colon is particularly sensitive to noradrenaline. No other tissue except rabbit's ear was so sensitive to this drug and no other drug was so active in this test. The threshold dose in the 2 c.c. bath was 5–20 mμg.

Fig. 4. Rat colon in 2 c.c. bath. Responses to acetylcholine (10 mμg. for 30 sec. every 2 min.) Inhibitory effects of adrenaline (*A*), noradrenaline (*N*) and α-ethylnor-adrenaline (*EN*). Doses in mμg.

Adrenaline was generally slightly less active than noradrenaline in this test. For example, Fig. 4 shows an experiment in which it was about half as active. This agrees with West's finding that some preparations made from the intestine are inhibited by noradrenaline in smaller doses than adrenaline. In the early part of some experiments, both of these drugs caused contraction of the muscle, but this effect always disappeared with further doses. It was surprising to find that histamine in suitable concentrations inhibited the response to acetylcholine. In higher concentrations histamine has been found to stimulate the rat's intestine (Feldberg & Schilf, 1930).

The interfering substances in control plasma caused contraction of this preparation and interfered with this test more than with that on the uterus.

These two tests on pieces of plain muscle from rats are an interesting contrast. The uterus provides the most sensitive and specific test for adrenaline, and the colon provides the most sensitive and specific test for noradrenaline. It is not known why these two drugs should act so differently in these two tests, but this fortunate circumstance provides a convenient method of discriminating between them. The intestines of guinea-pigs and rabbits were comparatively insensitive when used in this way. The reason why the rabbit's intestine was so insensitive is probably that it had been exposed to very unphysiological conditions in order to reduce the spontaneous activity.

It is known that when isolated intestine is studied in the conventional way, blood serum causes an initial inhibition followed by contraction. The inhibition is partly due to adenosine. The contraction is partly due to histamine, but mainly due to a substance which has not yet been identified (Gaddum, 1936). This unknown contractor substance may be the same as the 'slow reacting substance' of Feldberg & Kellaway (Kellaway, 1947). It has been found in platelets, but haemolysed red cells seem to be a richer source. In our experiments with heparinized human blood haemolysed red cells had marked actions like those of serum. The plasma, haemolysed platelets and haemolysed white cells had much feebler actions of the same kind.

Frog's heart. Various attempts have been made to use the isolated frog's heart for the detection of adrenaline in blood (Schlossmann, 1927). It may be very sensitive to adrenaline, particularly if it is beating feebly, and it has been suggested that it should be deliberately depressed in order to sensitize it. Schlossmann used aconitine for this purpose, but eventually abandoned this method. West (1947*b*) found that perfused frogs' hearts were rather insensitive to noradrenaline compared with adrenaline.

Frogs' hearts were used in the early stages of the present work. Straub's preparation gave satisfactory results with pure solutions of adrenaline and noradrenaline, though the ratio of activities was very variable. Cocaine (10^{-5}–10^{-6}) increased the sensitivity to adrenaline two to three times, but not that to noradrenaline. Ergotoxine antagonized adrenaline more effectively than noradrenaline. The heart was found to beat well in cat's plasma (heparin) diluted with half its volume of water, but in these conditions it was not sensitive to adrenaline.

It is well known that various substances in blood may affect the frog's heart. Adenosine compounds may be present in sufficient quantities to inhibit the heart, but the main effect of serum is generally stimulation (Clark, 1913). The nature of the substances with this effect is not known, but it is not lecithin, as was once supposed (Eggleton, 1926).

We have found that lysed human red cells are a good source of stimulant substances. Plasma or lysed white cells or platelets were less active. Lysed whole blood from rabbits or cats or man was also active. These solutions were dialysed against water in Verney's (1926) dialyser, and the dialysate had a complex effect which appeared to be due to two stimulant substances, both of which increased the rate and force of the beat of the frog's heart. The first substance had an immediate effect which often disappeared in the later stages of an experiment. The effect of the second substance was like that of adrenaline. It appeared after the first effect was over and did not become less with successive doses. It was found that the substances responsible for these effects could be removed from dialysates of haemolysed whole blood (from men, cats or rabbits) by adsorption on alumina of the type used by Shaw (1938). In these

experiments 5 c.c. of blood were mixed with 5 c.c. of water and dialysed against 10 c.c. of water for 4 hr. The pH of the dialysate was adjusted to 5–6 with HCl, using chlorophenol red as an indicator. The suspension of alumina (0·3–0·5 c.c./c.c. of dialysate) was then added and the mixture shaken, and then centrifuged. This removed the interfering substances which stimulate the frog's heart, but not adrenaline, which is only adsorbed on alumina at more alkaline reactions. Another coarser type of alumina was ineffective when used in the same way. This technique is similar to that used by Jørgensen (1945) for separating adrenaline from fluorescent substances in plasma. By such methods it should be possible to use the frog's heart for the estimation of adrenaline in blood. This test was, however, abandoned because a simpler method of obtaining inactive control samples was sufficient for tests on other tissues.

Rabbit's ear. Various previous workers have come to the conclusion that the best of the known tests for adrenaline in blood is the perfused rabbit's ear (Schlossmann, 1927; Kahlson & Werz, 1930; Kuré, Okinaka, Ohshima, Shimamota & Okamura, 1936). We have also been successful with this preparation. It could be used about 30 min. after starting the perfusion, and small doses of adrenaline continued to produce a brief decrease in the outflow for periods up to 72 hr. The sensitivity usually increased as the perfusion continued, and reached a maximum on the second day, when the preparation was occasionally ten times as sensitive as it had been. It has often been found convenient to make such preparations the day before they were needed. When a series of doses of adrenaline were injected at regular intervals the sensitivity increased for 30–60 min., but this increase of sensitivity was lost when the series of injections ceased.

In the later stages of a perfusion the response appeared to depend on the concentration of calcium in the fluids injected. Small doses of adrenaline in saline became less effective than the same doses in Locke solution. The use of a perfusion fluid with a low calcium content caused an increase of sensitivity, but the effect was small and was not further investigated. It was hoped to eliminate possible interference from histamine by using neoantergan in the perfusion fluid (Dews & Graham, 1946). This was unsatisfactory since this drug inhibited the actions of adrenaline and noradrenaline, as well as those of histamine, though less potently. When small doses of ergotoxine ethane sulphonate (0·1 μg.) or dihydroergotamine (0·05 μg.) were injected into the cannula they caused a temporary inhibition of the effects of similar doses (by weight) of adrenaline and noradrenaline, both of which were equally affected. Noradrenaline was usually slightly less active than adrenaline. Fig. 5 shows an experiment in which the ratio of activities was between 1 and 2.

Cat's plasma in doses greater than 0·1 ml. caused vaso-constriction in this preparation if the cooling and centrifuging of the blood had been too slow. This

effect was not due to histamine since it was not antagonized by neoantergan. On the other hand, the surprising observation was made that suitable small doses of dihydroergotamine abolished this effect of plasma while leaving the effect of adrenaline unchanged.

The fact that ergot alkaloids inhibit the action of a vaso-constrictor substance which may be present in defibrinated blood was established by Heymans, Bouckaert & Moraes (1932). This substance is presumably different from the substance studied by Kahlson & Werz (1930) the effects of which were not antagonized by ergotoxine.

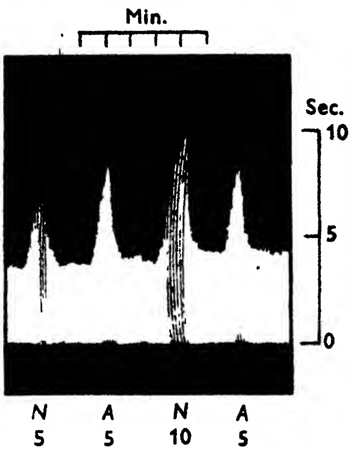


Fig. 5.

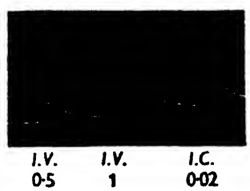


Fig. 6.

Fig. 5. Outflow from perfused rabbit's ear. Heights measure drop interval. Noradrenaline and adrenaline. Doses in mμg.

Fig. 6. Cat. Nictitating membrane. Intravenous (*I.V.*) and intracarotid (*I.C.*) injection of noradrenaline. Doses in μg.

Other perfused tissues. None of the other perfused tissues was as sensitive as the rabbit's ear. Whole frogs were slightly more sensitive than frogs' hind limbs, but too erratic. Dialysates of haemolysed whole blood from cats or men caused vaso-constriction in perfused frog's vessels (0·5 c.c. of 1/10 dilution). Plasma separated by rapid centrifugation usually had no effect even when undiluted.

Nictitating membrane. This tissue has been much used as an indicator of substances liberated by adrenergic nerves into the general circulation. The effective dose by intravenous injection has been found to be twenty-five to fifty times the effective dose by intra-arterial injection (Fig. 6), and the latter technique was therefore used. When heparinized whole blood, which had been standing in glass, was injected it usually caused a contraction of the membrane. Plasma which had been separated with the precautions recorded above usually

had little or no effect in doses of 0·5 c.c. injected rapidly, except in conditions where the effect could reasonably be attributed to adrenaline or noradrenaline.

Perfusion in situ. This technique provides a simple means of comparing the effects of drugs on different parts of the circulation. The cat's spleen responded to smaller doses of adrenaline than the tissues supplied by the superior or inferior mesenteric artery, the renal, hepatic or femoral artery. The activity of noradrenaline was usually about equal to that of adrenaline (Fig. 7) or slightly greater and sometimes as much as twice as great. When the femoral artery or the artery to the tail was perfused in cats anaesthetized with ether, adrenaline usually caused a fall of pressure while noradrenaline caused a rise.

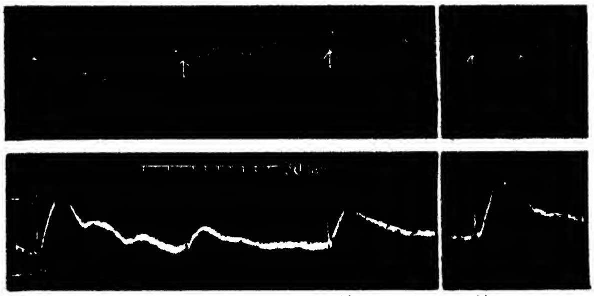


Fig. 7. Cat. Spleen perfused *in situ*. Splenic volume (above) and perfusion pressure (mm. Hg). Injection of adrenaline (*A*) and noradrenaline (*N*). Doses in mμg.

The difference between adrenaline and noradrenaline. In many tests the action of noradrenaline was about equal to that of adrenaline. It was generally slightly less active, but in some tests it was slightly more active. No tissue is known on which noradrenaline has much more effect than adrenaline, but various tissues are known on which it has much less action. Barger & Dale (1910) found that it had much less inhibitor action on the virgin cat's uterus and on the arterioles of cats. Its inhibitory action on the uteri of rats and guinea-pigs is also weak. These facts have suggested that the essential difference between these two drugs is that noradrenaline lacks inhibitory actions, but West (1947*b*) has drawn attention to various exceptions to this generalization. For example, the inhibitor action of noradrenaline on the intestine is often greater than that of adrenaline, and its excitor action on perfused frog's heart is much less.

Solutions of the pure substances can easily be distinguished by using a test in which noradrenaline is relatively active in parallel with one in which it is less active. The specific tests, in which noradrenaline is relatively inactive, are those showing high values in the last column of Table 2 such as the biological tests on the uterus and the perfused frog's heart, the fluorescence test (Gaddum & Schild, 1933) and the colorimetric test of Shaw (1938). If adrenaline and

noradrenaline are both present together in similar amounts any of these latter tests can be used to estimate adrenaline without much interference from noradrenaline. There is no simple way of estimating noradrenaline in the presence of similar amounts of adrenaline.

Other substances. Five sensitive tests were selected for more detailed investigation. The effects of a number of substances in these tests are summarized in Table 3 which shows the ratios of the doses of these substances to the equivalent doses of adrenaline. These ratios were found to vary fairly widely. This variation does not diminish the value of parallel quantitative assays for qualitative identification.

TABLE 3. Ratio of doses of various drugs to equivalent dose of L-adrenaline

Effect	Inhibitor			Excitor	
Animal	Rat		Rabbit	Cat	
Organ	Uterus	Colon	Ear	Spleen	Nictitating membrane
Dose adrenaline (mμg.)	0·5	2–5	2	30	10–50
Probable precursors of adrenaline:					
Hydroxytyramine	2000–6000	10^5	(3000)	75	100
L-Noradrenaline	75–300	0·2–1	1–3	0·5–1	1
α-Substituted noradrenalines:					
DL-corbasil	10	5	1000–5000	—	200
DL-α-ethylnoradrenaline	50, 60	0·5	20	1·5	7, 15
β-Substituted adrenalines:					
Epinine	120	100	20	10	1·3
Adrenalone	25	4, 20	130, 200	200	150
N-Substitution:					
DL-N-methyladrenaline	850	60	40	—	25
DL-N-ethylnoradrenaline	0·5, 1	1	12	Dilator	45
DL-N-isopropylnoradrenaline	0·5, 1	1	10^4	Dilator	Inhibitor
Other substances:					
Tyramine	3×10^5, 10^6	10^5	—	250	400
Ephedrine	5000	750	—	10^4	—
Histamine	5000	50	10	Dilator	10
Adenosine	Excitor	3000	—	—	—

On the colon hydroxytyramine is no more active than tyramine. On the other tissues it is somewhat more active, but still not really potent. The introduction of the β-OH converts it to noradrenaline and greatly increases its activity in all the tests and especially on the colon. The great effect of the β-OH is also shown by comparing adrenaline and epinine. Of both these comparisons it may be said that reduction of the OH to H decreases the activity. On the other hand, oxidation of this group in adrenaline to form the ketone adrenalone also decreases the activity. By comparing the figures for epinine and adrenalone it may be seen that reduction appears to affect the inhibitor actions more than the excitor actions and oxidation appears to affect the excitor actions more than the inhibitor actions.

The replacement of the N-methyl group in adrenaline by larger alkyl groups appears to have little effect on the inhibition of the uterus and colon, but diminishes or reverses the excitor effects. These compounds have been studied by Konzett (1940) and found to have powerful dilator effects on blood vessels and bronchi.

Discrimination between different substances. In the case of most of the substances in Table 3 the ratios shown in the different tests vary widely and there should be little difficulty in distinguishing these substances from adrenaline. In the case of the two N-mono-alkyl compounds the ratios on the rat's uterus and colon were about equal, but these compounds are distinguished by their lack of excitor effects.

α-Ethylnoradrenaline showed ratios not very different from those of noradrenaline, but the two substances could presumably be distinguished by using the rat's uterus or colon together with the rabbit's ear or the nictitating membrane.

A detailed comparison of the other figures shows that there should be no difficulty in distinguishing any one of these substances from any other.

DISCUSSION

If parallel quantitative assays of an unknown solution are made by the five tests shown in Table 2, using adrenaline as a standard, and if these tests agree with a maximum error ratio of not more than 2, it is reasonable to conclude that the effects of the unknown solution are due to adrenaline. It is quite certain that they are not due to any one of the other substances used in the present investigation. Any one of these other substances can also be identified in the same way, provided that only one pharmacologically active substance is present in the solution.

This principle of parallel quantitative assays was used by Cannon & Rosenblueth (1937) to prove that adrenaline is not the only substance liberated by adrenergic nerves. Such negative evidence is immediately convincing. Positive evidence identifying an unknown substance is only convincing when it is known that the tests used can distinguish closely allied substances from one another. The tests described here do appear to have this power. They have been applied to the study of the substances released by the adrenergic nerves in the cat's spleen and the results are published elsewhere (Peart, 1949).

SUMMARY

1. Various pharmacological methods of estimating adrenaline and allied substances have been compared and five sensitive methods selected for detailed study.

2. Closely allied sympathomimetic amines can be distinguished from one another by making parallel quantitative assays by these five methods.

These substances can therefore be identified by such assays in unknown solutions.

3. When these tests are applied directly to blood the result is vitiated by the release of interfering substances. This can be avoided if the blood is collected with much heparin in cooled tubes coated with silicone. If the cells are rapidly removed by centrifugation adrenaline (10^{-8}) or noradrenaline (10^{-7}) can be identified in the plasma, if present.

We are much indebted to Dr M. L. Tainter for samples of the various compounds made by Sterling Winthrop, and to Dr H. Blaschko for samples of hydroxytyramine and methyladrenaline.

REFERENCES

Bain, W. A., Gaunt, W. E. & Suffolk, S. F. (1937). *J. Physiol.* **91**, 233.

Barger, G. & Dale, H. H. (1910). *J. Physiol.* **41**, 19.

Barsoum, G. S. & Gaddum, J. H. (1935). *J. Physiol.* **85**, 1.

Cannon, W. B. & de la Paz, D. (1911). *Amer. J. Physiol.* **28**, 64.

Cannon, W. B. & Rosenblueth, A. (1937). *Autonomic Neuro-Effector Systems.* New York: The Macmillan Co.

Clark, A. J. (1913). *J. Physiol.* **47**, 66.

Dews, P. B. & Graham, J. D. P. (1946). *Brit. J. Pharmacol.* **1**, 278.

Eggleton, P. (1926). *Biochem. J.* **20**, 395.

Feldberg, W. & Schilf, E. (1930). *Histamin.* Berlin: Springer.

Gaddum, J. H. (1936). *Gefässerweiternde Stoffe der Gewebe.* Leipzig: Thième.

Gaddum, J. H. & Kwiatkowski, H. (1938). *J. Physiol.* **94**, 87.

Gaddum, J. H. & Schild, H. (1933). *J. Physiol.* **80**, 9*P*.

Heymans, C., Bouckaert, J. J. & Moraes, A. (1932). *Arch. int. Pharmacodyn.* **43**, 468.

Hoskins, R. G. (1911). *J. Pharmacol.* **3**, 93.

Isola, W. & Bacq, Z. M. (1946). *Arch. int. Physiol.* **54**, 30.

de Jalon, P. G., Bayo, J. B. & de Jalon, M. G. (1945). *Farmacoterap. Actual.* **2**, 313.

Jørgensen, K. S. (1945). *Acta pharmacol.* **1**, 225.

Kahlson, G. & v. Werz, R. (1930). *Arch. exp. Path. Pharmak.* **148**, 173.

Kellaway, C. H. (1947). *Edin. med. J.* **54**, 333.

Konzett, H. (1940). *Arch. exp. Path. Pharmak.* **197**, 27, 41.

Kuré, K., Okinaka, S., Ohshima, K., Shimamota, T. & Okamura, D. (1936). *Klin. Wschr.* **15**, 477.

Luduena, F. P., Ananenko, E., Siegmund, O. H. & Miller, L. C. (1949). *J. Pharmacol.* **95**, 155.

Peart, W. S. (1949). *J. Physiol.* **108**, 492.

Reid, G. & Bick, M. (1942). *Med. J. Aust.* **1**, 245.

Richards, A. N. & Plant, O. H. (1915). *J. Pharmacol.* **7**, 485.

Schlossmann, H. (1927). *Arch. exp. Path. Pharmak.* **121**, 160.

Shaw, F. H. (1938). *Biochem. J.* **32**, 19.

Verney, E. B. (1926). *J. Physiol.* **61**, 319.

West, G. B. (1947*a*). *Brit. J. Pharmacol.* **2**, 121.

West, G. B. (1947*b*). *J. Physiol.* **106**, 418, 426.

Zucker, M. B. (1944). *Amer. J. Physiol.* **142**, 12.

J. Physiol. (1949) 108, 482–490 615.783.22:612.464.1.547.262

THE EFFECT OF NICOTINE ON THE DIURESIS INDUCED BY ETHYL ALCOHOL

By M. GRACE EGGLETON

From the Department of Physiology, University College, London

(*Received* 13 *September* 1948)

In an attempt to obtain further evidence as to the mechanism of the diuresis induced by the ingestion of ethyl alcohol (ethanol), advantage was taken of some results published by Burn, Truelove & Burn (1945) on the antidiuretic action of nicotine on water diuresis. These workers concluded that the inhibition of the diuresis produced either by smoking cigarettes or by intravenous injection of nicotine was probably due to stimulation of the supraoptic nucleus, thereby leading to a discharge of antidiuretic hormone from the post-pituitary gland. They also came to the tentative conclusion that the degree of inhibition was correlated with the smoking habits of the different subjects, the heavy smoker being less sensitive than the light to a given dose of nicotine.

Since earlier work on alcohol diuresis had led to the conclusion that this diuresis resulted from the depressant action of alcohol on the hypothalamic centres controlling the output of antidiuretic hormone, it was surmised that nicotine might be expected to inhibit this diuresis also. The matter was put to the test on a relatively large number of subjects, several of them complete non-smokers.

METHODS

In the main group of student subjects on whom experiments were first conducted, the nicotine was given subcutaneously 5–15 min. before ingestion of the alcohol. The course of the diuresis and total output of urine in 2·5 hr. was then compared in each subject with that resulting from ingestion of the same dose of alcohol without nicotine. In the majority of subjects the two experiments were carried out at an interval of 7 days. It is known that many factors, particularly external temperature, affect the diuretic response to a given dose of alcohol in the same individual (Eggleton, 1942), and this source of error was eliminated as far as possible by the use of paired experiments: one subject received alcohol alone and the other nicotine plus alcohol on the same experimental day. The general regime in all experiments was that followed in earlier work; they were performed in the afternoon, lunch having been omitted, and a glass of water taken 2–2½ hr. before the beginning of the experiment. In some later experiments, the time of injection of the nicotine was varied, for reasons which are given later in the text, and in a few the nicotine was given intravenously.

Sensori-motor test. In earlier work, typing had been found as reliable a test as any for the detection of changes induced by alcohol in the higher nervous centres (Eggleton, 1941), and this was again used as a guide to the rate of absorption of the alcohol. The test passage was shorter and simpler and the establishment of a steady base-line performance attained correspondingly sooner.

Creatinine determination. This was made in the usual way, by colorimetric comparison with suitable standards of the alkaline picrate; the use of a Wratten filter no. 74 greatly increased the accuracy of the comparisons. In later experiments, a simple photoelectric colorimeter was used.

RESULTS

A successful comparison of the diuretic responses to alcohol alone, and to alcohol plus nicotine, was made in twenty-two subjects, twelve smokers and ten non-smokers. The results on twelve others were discarded for various reasons: nausea or even vomiting, difficulty in emptying the bladder, or extreme variations in external temperature on the two experimental days.

TABLE 1. The effect of nicotine on the diuretic response to alcohol in smokers and non-smokers

Subject	Body weight (kg.)	Cigarettes per day	Time of injection of nicotine (min.)	Diuretic response to Alcohol (c.c./2½ hr.)	Diuretic response to Alcohol + nicotine (c.c./2½ hr.)	Percentage change in diuretic response
G ♀	70	0	−10	810	875	+ 8
F ♂	71·5	0	+10	•555	600	+ 8
(1) Sm ♀	61	0	− 1	600	655	+ 9
B ♀	63	0	− 5	710	790	+ 11
U ♀	52	0	− 8	455	505	+ 11
(2) W ♂	63·5	0	− 8	740	975	+ 31
(2) W ♂	63·5	0	− 1	645	870	+ 35
F ♂	71·5	0	− 7	645	875	+ 36
(1) Sc ♂	67·5	0	+ 7	280	390	+ 39
I ♂	63	0	− 9	255	370	+ 45
Ca ♂	62	0	+12	335	800	+140
H ♀	52·5	10–15	− 7	595	600	0
A ♀	63·5	1	− 5	830	780	− 6
K ♂	73·5	5	− 7	500	470	− 6
R ♀	59	4–5	−11	655	535	− 18
(2) Ri ♂	62	10–20	− 1	745	580	− 22
(1) D ♂	66	25–50	+ 7	555	415	− 25
M ♂	68	7–8	−10	710	440	− 38
(3) E ♀	52·5	30–40	− 8	660	400	− 39
Q ♂	70·5	Occasional	− 5	655	375	− 43
J ♀	82·5	1	−25	960	500	− 48
L ♂	66	0–7	−12	345	175	− 49
V ♂	63·5	2	−15	350	60	− 83

The same dose of alcohol was given on both occasions: 32 g. in 200 c.c. in all subjects except (1) 24 g., (2) 40 g. in 250 c.c., (3) 50 g. in 250 c.c. In all subjects 1 mg. nicotine (as tartrate) was injected subcutaneously, timed in relation to the beginning of the drink.

The unexpected effect seen in Table 1 was obtained. No diminution in total diuresis was observed in any of the non-smokers, and in several a significant increase; whereas, in all but one of the smokers, nicotine caused a decreased urine output of from 6 to 83 %. Although nausea leads to inhibition of either a water or an alcohol diuresis, the temporary feeling of 'dizziness' noted by many subjects following the injection of nicotine produced no such effect and was not responsible for the difference in diuretic response of the two groups; it occurred in five of the non-smokers and in six of the smokers.

Three possible causes of the difference in diuretic response were considered. First, the possible though rather improbable explanation that nicotine delays the rate of absorption of alcohol in the non-smoker and enhances it in the smoker, for it is known that a given dose of alcohol results in a larger diuresis if slowly absorbed (Eggleton, 1942). In the absence of facilities for determining blood alcohol concentration directly, advantage was taken of the fact that the higher nervous centres are more affected during rapid than during slow absorption (Eggleton, 1941). The typing test was used, therefore, to assess the

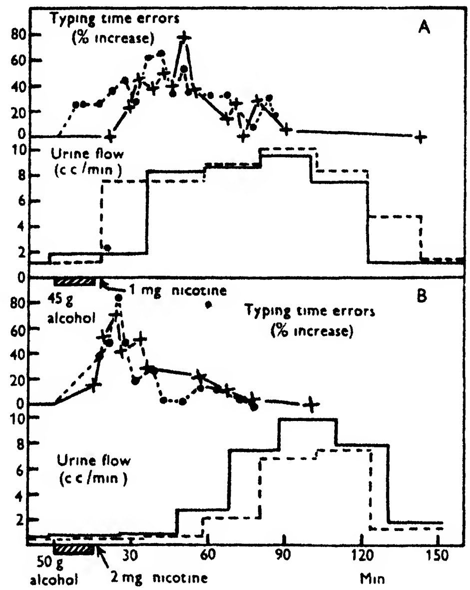


Fig. 1. Effect of nicotine in reducing the diuretic response in a smoker (B) and enhancing it in a non-smoker (A) although the rate of absorption of alcohol, as judged by its action on the higher nervous centres, was neither increased in the one (B) nor decreased in the other (A). +——+, alcohol alone; •----•, alcohol + nicotine.

effect of nicotine on alcohol absorption in a smoker and in a non-smoker. In the smoker (Fig. 1 B), normally a rapid absorber, the nicotine caused no apparent change in rate of absorption as judged by rate of onset and degree of upset of the higher centres, yet the diuretic response was appreciably reduced. In the non-smoker (Fig. 1 A), normally a slower absorber, the rate of absorption was initially faster on the occasion when nicotine was given and yet the diuretic response was greater.

A second possible cause of the different diuretic response in smoker and non-smoker might be a differential action of nicotine on the renal vessels: a constriction of the vas afferens in the smoker, giving a reduction in filtration rate, and a constriction of the vas efferens in the non-smoker, giving the reverse effect.

It is known that the total output of creatinine affords a reasonably accurate method of determining changes in filtration rate in short-term experiments of this nature (Blumgart, Gilligan, Levy, Brown & Volk, 1934) and was accordingly used to test the theory outlined above. In eight experiments on one subject the validity of the method was clearly demonstrated. On each occasion the resting creatinine output lay between 40½ and 44½ mg./hr. and the final post-diuretic value (after 2½ hr.) between 38 and 43 mg./hr. Any change of less than 10% was, therefore, not regarded as significant. In three experiments in which alcohol alone was taken, the overall change in creatinine output during the following 2½ hr. varied from −2 to +11% of the resting value; and in four experiments of a similar nature, in which nicotine was injected, the change varied from 0 to +4½%. It is clear, therefore, that the reduced diuretic response is not due to a reduction in filtration rate in this subject. A single pair of experiments was made on each of six other subjects and the results shown in Table 2 indicate that neither the reduction of diuretic response in smokers nor its enhancement in non-smokers by nicotine can be attributed to changes in filtration rate. Further evidence in support of this conclusion is given in later figures.

TABLE 2. Showing the lack of effect of nicotine on the creatinine output during alcohol diuresis

	Alcohol alone		Alcohol + nicotine		Effect of nicotine on	
Subject	Diuresis (c.c. urine/2½ hr.)	Change in creatinine output on resting value (%)	Diuresis (c.c. urine/2½ hr.)	Change in creatinine output on resting value (%)	Diuretic response (c.c.)	Creatinine output (%)
Non-smokers:						
Sc	280	+ 1	390	+ 1	+110	0
Ca	335	+ 7	800	+ 4	+465	− 3
W	645	+ 4	870	+ 4	+225	0
F	555	− 6	600	−16	+ 45	−10
Smokers:						
D	555	+14	410	+18	−145	+ 4
Ri	745	+ 6	580	− 4	−165	−10

The third possible cause of difference in diuretic response of smokers and non-smokers is that nicotine stimulates the output of antidiuretic hormone in the former, but not in the latter. The reasons for considering such an unlikely hypothesis are given in the Discussion; here, it is sufficient to mention that such an interpretation derives strong support from the fact that the lag in onset of diuresis—attributable to antidiuretic hormone still in circulation—is not affected by nicotine in any of the non-smokers, but in nine of the twelve smokers examined was considerably increased. The results shown in Fig. 2 illustrate this effect, which is apparent also, though to a lesser degree, in the results shown in Fig. 1 B. The fact that the inhibitory action of nicotine is not

due to any reduction in filtration rate is also emphasized in Fig. 2. The increase in creatinine output resulting from ingestion of alcohol is more marked and prolonged in this subject than in most, but is enhanced rather than diminished by the nicotine injection.

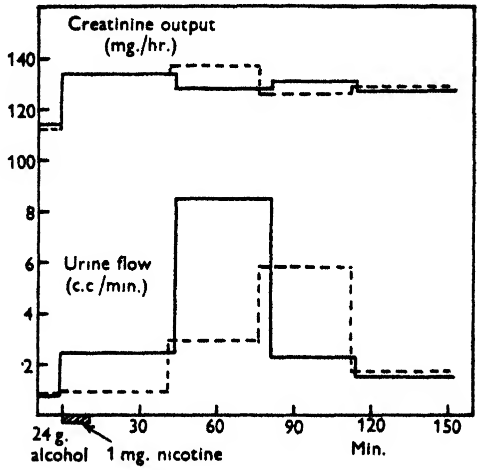


Fig. 2. Effect of nicotine in increasing the lag in onset of alcohol diuresis in a smoker, an effect never observed in non-smokers. ——, alcohol alone; – – – –, alcohol + nicotine.

Comparison of the action of nicotine on water diuresis and on alcohol diuresis in smokers and non-smokers

In view of the apparent discrepancy between these results and those obtained by Burn *et al.* (1945) on the effects of nicotine on water diuresis, further experiments were performed to test the response of both water diuresis and alcohol diuresis to nicotine in the same individuals. Only one completely controlled set of experiments was successful, owing to unduly large variations in external temperature, but in four other subjects nicotine caused a depression of water diuresis of 265 and 95 c.c. respectively in the smokers and an enhancement of 120 and 540 c.c. in the non-smokers. The results of the complete experiment are given in Table 3. One non-smoker was paired with one smoker on the first 2 days and with another on the second. The smokers responded to nicotine by a depression of both water and of alcohol diuresis under conditions in which the non-smoker showed an enhanced diuresis.

Although it seemed unlikely that nicotine would produce completely different effects when its site of injection was varied, a few experiments comparable with those of Burn *et al.* were made, the nicotine being given intravenously. The results presented in Fig. 3 show clearly that the non-smoker still responds with an enhancement of water diuresis, whereas the smoker shows the typical inhibition observed by Burn *et al.* The smoker of this experiment would be defined as a light smoker; he took 3 or 4 pipes a few times each year. When the

experiment was repeated on a heavy smoker (thirty to forty cigarettes daily), the inhibition of water diuresis by 1 mg. nicotine was negligible; 2 mg., however, produced a more prolonged inhibition (70 min.) than had 1 mg. in the light smoker. Nicotine given intravenously appears to cause a reduction in filtration

TABLE 3. Showing the opposing action of nicotine on both alcohol diuresis and water diuresis in the smoker and non-smoker

(All values are expressed as c.c. urine in $2\frac{1}{2}$ hr.)

	2. ix. 47 Temp. 18·5° C. 24 g. alcohol	4. ix. 47 Temp. 19·0° C. 24 g. alcohol + 1 mg. nicotine	Effect of nicotine
Non-smoker	280	390	+110
Smoker	555	410	−145
	23. ix. 47 Temp. 23·0° C. 560 c.c. H_2O	25. ix. 47 Temp. 22·5° C. 560 c.c. H_2O + 1 mg. nicotine	
Non-smoker	205	395	+190
Smoker	600	545	− 55

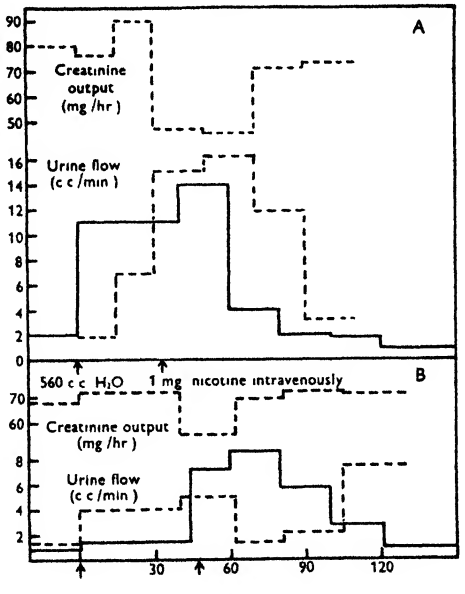


Fig. 3. Effect of an intravenous injection of nicotine on a water diuresis, in the non-smoker (A) enhancing the diuretic response, and in the smoker (B) inhibiting the diuresis. ——, water alone; – – – –, water + nicotine.

rate as judged by changes in creatinine output, but such action is clearly not the cause of the inhibition of diuresis. The action was most obvious in the non-smoker, at the height of diuresis, and in the smoker preceded the inhibition of urine flow.

DISCUSSION

The main purpose of the research, an attempt to establish a further link in the chain of evidence identifying alcohol diuresis with water diuresis, has been attained. Those subjects in whom nicotine causes a diminution in the diuretic response to water show also a reduced diuretic response to alcohol.

The unexpected finding that such an inhibition of either type of diuresis is not observed in complete non-smokers appears to be at variance with the tentative conclusion drawn by Burn *et al.* (1945). However, all of the seven subjects studied by these authors were smokers and only two experiments are quoted in which nicotine was injected, the effect in the remainder being due to rapid smoking with deep inhalation of one to three cigarettes. These results cannot be accepted without reserve; for the light smoker would be more likely to suffer ill-effects (nausea and even vomiting occurred in some subjects) than the heavy smoker: and such ill-effects, whatever tneir cause, lead to an inhibition of either water or alcohol diuresis.

The one comparison made in this research between a heavy and a light smoker with an intravenous injection of nicotine appears to support the conclusion of Burn *et al.*, but there is no more than a hint of such a correlation in the group of smokers given the drug subcutaneously. One variable factor, not recognized at the time, may have been responsible for obscuring any clear-cut relationship between smoking habits and degree of inhibition by nicotine, if such exists. The rapidity of absorption of a subcutaneous injection appears to vary considerably with the amount of subcutaneous fat. One subject who showed a well-marked inhibition with the nicotine injected 10 min. before taking alcohol was tested a year later when the body weight had diminished by 8%. The injection was now quite ineffective, but if given later (from 20 to 60 min. after beginning the alcohol drink), produced the usual degree of inhibition. With body weight regained after another year, nicotine given 5–10 min. before the alcohol was again effective. The group of subjects used varied considerably, from definitely obese (subject J) to very lean (subjects H and R), and it is not improbable that in the latter group the major action of the nicotine had passed before the alcohol diuresis was induced. The total evidence available at the moment, however, would seem insufficient to permit any sure conclusion to be drawn concerning the establishment of different degrees of tolerance to nicotine within the group of smokers.

The cause of the difference in response to nicotine of the smokers and non-smokers must remain at present a matter of conjecture. It is not due to a differential action on the rate of absorption of alcohol in the two groups, nor to changes in glomerular filtration rate. The increased lag in onset of the diuresis in all subjects in which a pronounced inhibition occurred suggests that this is due to an increased output of antidiuretic hormone. Nicotine is known

to stimulate both sympathetic and parasympathetic systems, and it seems not unlikely that subjects addicted to nicotine might respond to a single injection of the drug differently from those whose tissues had not previously been exposed to its action. If this postulate be accepted, other evidence confirms the probability that the post-pituitary gland would react differently in the two groups in regard to its output of antidiuretic hormone. Verney (1946) has shown that sympathetic stimulation antagonizes the action of emotional stimuli in causing output of antidiuretic hormone, and it seems permissible from his evidence to consider the supraoptic nuclei as part of the parasympathetic system. The opposite view put forward by Hare (1946), that 'the supraopticohypophyseal system seems to be established as an autonomic effector, *comparable in innervation and function to the adrenal medulla*', is apparently based on the fact that this system is stimulated by acetylcholine (Pickford, 1939); the evidence can obviously be used in support of either view. If the first action of nicotine is on the sympathetic system, one would not expect any increase in output of antidiuretic hormone, but rather a decrease. This situation is met in the non-smoker. In those made tolerant to nicotine by habitual smoking, one may readily suppose a second action of the nicotine injection becoming apparent on the parasympathetic system: thus, stimulation of the supraoptic nuclei, with consequent inhibition of the diuresis.

The recent suggestion of Trueta, Barclay, Daniel, Franklin & Prichard (1947) that the inhibition of urine flow observed by Verney and by Burn *et al.* was due to a diversion of blood flow from renal cortex to medulla rather than to liberation of antidiuretic hormone is not supported by the results presented here. Since the main site of filtration is supplied by the cortical circulation, any diversion of blood from cortex to medulla will involve a reduction in filtration. This state of affairs may be of normal occurrence in the rabbit, with which Trueta *et al.* were mainly concerned, for it is known to differ from most other mammals (including man) in showing a consistent variation in filtration rate with variation in rate of urine flow during a water diuresis. In man, however, if creatinine output be accepted as a guide to changes in filtration rate, it is clear that the changes in rate of urine flow induced by nicotine are unconnected with changes in filtration rate.

SUMMARY

1. Subcutaneous injection of 1 mg. nicotine (as tartrate) a few minutes before or after ingestion of alcohol caused a diminution in diuretic response in eleven out of twelve smokers. No such diminution was observed in ten non-smoking subjects, in four of whom a significant increase occurred (Table 1).

2. This difference in response of the two groups to the action of nicotine could not be attributed to its differential effect on the rate of absorption of the alcohol (Fig. 1), nor on the glomerular filtration rate (Table 2).

3. One possible explanation of this difference is a differential action of nicotine on the sympathetic and parasympathetic systems in the two groups; stimulation of the sympathetic system is known to antagonize the output of antidiuretic hormone (the non-smoking group), whereas in the smokers it appeared to cause an increased output of this hormone as indicated by an increased lag in onset of the diuresis (Fig. 2).

4. This hypothesis is supported by the observations: (*a*) that the reaction of any subject to nicotine is identical whether water or alcohol is used as the diuretic agent, smokers showing a diminution and non-smokers an enhancement of the diuretic response (Table 3); and (*b*) that this difference in response of smoker and non-smoker is still observed when the drug is given intravenously during a water diuresis (Fig. 3).

REFERENCES

Blumgart, H. L., Gilligan, D. R., Levy, R. C., Brown, M. G. & Volk, M. C. (1934). *Arch. intern. Med.* **54**, 40.

Burn, J. H., Truelove, L. H. & Burn, I. (1945). *Brit. med. J.* **1**, 403.

Eggleton, M. G. (1941). *Brit. J. Psychol.* (General Section), Part I, **32**, 52.

Eggleton, M. G. (1942). *J. Physiol.* **101**, 172.

Hare, K. (1946). *Ann. Rev. Physiol.* **8**, 395.

Pickford, M. (1939). *J. Physiol.* **95**, 226.

Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, J. K. & Prichard, M. M. L. (1947). *Studies of the Renal Circulation.* Oxford: Blackwell Scientific Publications.

Verney, E. B. (1946). *Lancet*, **2**, 781.

J. Physiol. (1949) 108, 491–501 577·174·5·612·418

THE NATURE OF SPLENIC SYMPATHIN

By W. S. PEART

From the Department of Pharmacology, University of Edinburgh

(*Received* 13 *September* 1948)

The substance liberated on stimulation of the adrenergic nerves to various organs has been much studied, and in cases where a direct assay of the perfused fluid has been made, the fluid has always been found to contain adrenaline (Loewi, 1936; Gaddum & Kwiatkowski, 1939; Gaddum, Jang & Kwiatkowski, 1939; Bülbring, 1944). The perfused fluid has always been a simple salt solution. The methods used to identify the substance released on nervous stimulation when the normal circulation of the animal is intact have been indirect, and involve qualitative inferences from the effect on other organs in the same animal (Cannon & Rosenblueth, 1937; Greer, Pinkston, Baxter & Brannon, 1938). In this paper the term sympathin is used in Euler's sense (1946*b*) to denote the substance released on stimulation of the adrenergic nerves, without the special implications involved in the use of the term by Cannon & Rosenblueth. The evidence has pointed to the release of a sympathin with mainly excitor properties from certain organs, e.g. the liver, and another with mainly inhibitor properties from other organs, e.g. the intestine (Cannon & Rosenblueth, 1937). From other organs effects interpreted as due to a mixture of the two sympathins were obtained. Gaddum & Goodwin (1947) pointed out that these methods can never give precise information, as the rate of release of the sympathin is unknown. They showed, with intravenous injections of adrenaline given at varying rates, the importance of this factor in modifying tissue responses, and the conclusions which could be safely drawn. It therefore seemed that the method which would give most information was that of direct assay of the venous blood collected from an organ before and after stimulation of its adrenergic nerves. The cat's spleen has proved to be suitable for this purpose. Previous work on the effects of the stimulation of the nerves to the spleen has been mainly concerned with the physical effects, such as the decrease in volume (Schäfer & Moore, 1896; Barcroft, Nisimaru & Puri, 1932). However, Bacq & Fredericq (1935) demonstrated the release into the cat's blood stream of a substance which caused contraction of the sensitized nictitating membrane. This effect, like that of adrenaline, could be abolished by the use of the dioxane

derivative 933F. Euler (1946*a*, *b*) found that the spleen and splenic nerves in the horse and cow contain an adrenaline-like substance which is not adrenaline. In the case of the splenic nerves this could not be distinguished from noradrenaline. For lack of this substance the splenic extracts had to be compared with dihydroxynorephedrine (corbasil), with which there was great similarity. This work has been recently confirmed by Bacq & Fischer (1947), using noradrenaline uniformly for comparison. They further claim that in certain splenic nerve extracts there appears to be a mixture of substances, predominantly noradrenaline with some adrenaline. Holton (1949) has given evidence that an extract of a human adrenal medullary tumour (chromaffinoma) contained a mixture of noradrenaline and adrenaline. An account of the methods used for the recognition and estimation of sympathomimetic amines in blood is published in a separate paper (Gaddum, Peart & Vogt, 1949), in which the use of parallel quantitative assays for this purpose is discussed.

METHODS

Collection of splenic blood. Cats anaesthetized with ether and pentobarbitone (Nembutals Abbott) or chloralose, were used. Many had cocaine (8 mg./kg.) at the beginning of the experiment. The splenic nerves were dissected free from the artery and divided centrally. Vascular connexion of the spleen with the stomach and greater omentum were divided between ligatures. Splenic venous blood was led from a cannula in the splenic vein through rubber tubes and a small closed glass reservoir, to either the left external jugular vein or the femoral vein. The reservoir, in which an air space was maintained, showed the rate of flow. A glass T-piece formed part of the circuit leading from the splenic venous cannula, and the free arm carried a short rubber tube, closed by a clip. The method of collection was to clamp the tube leading to the reservoir and open the clip on the side-arm of the T tube. Stimulation of the nerves was through platinum electrodes using a Ritchie Sneath stimulator to provide alternating current (50 cycles per sec.) of 10 V. potential, which was optimal. In more than half the experiments the suprarenals were excluded from the circulation by ligatures. Heparin (1000 units per kg.) was injected intravenously, and in the later experiments heparin (10 units per ml. of blood) was in the tube in which the blood was collected. All glass connexions were treated with silicone (General Electrical Co., Drifilm 9987). The blood was collected into siliconed tubes standing in ice, and the plasma was separated by centrifugation in ice at 3000 r.p.m. for 5 min.

Procedure. Usually, but not always, the spleen was first partly emptied of blood by stimulation of the nerves, as otherwise the sample collected after stimulation contained too high a proportion of cells, and too little plasma for assay purposes. After 5–20 min. a control sample of splenic blood was collected, followed by stimulation of the nerves, usually for 1 min. Splenic blood was collected during and following this period. An arterial sample was collected during or at the end of splenic sampling. The maximum volume of blood in each sample was usually 4–6 ml.

Assay methods. Samples of plasma collected with the above precautions were used in five pharmacological tests, without further treatment. The tests are described in detail elsewhere (Gaddum *et al.* 1949). The best of these tests for adrenaline is that which uses its antagonism to acetylcholine as described by de Jalon, Bayo & de Jalon (1945). A virgin rat's uterus, kept at 30° C. in Locke solution with low calcium content, is stimulated at 2 min. intervals with acetylcholine. Adrenaline added 1 min. before the acetylcholine inhibits in part the next response. A similar technique applied to the rat's colon gives a sensitive test for noradrenaline. Rabbits' ears were perfused by the method of Gaddum & Kwiatkowski (1938). Drugs and plasma were injected into the arterial cannula. The height of the record shows the interval between drops in the outflow using a Gaddum

recorder. Contractions of the nictitating membrane (Cannon & Rosenblueth, 1937), or lower eyelid of cats (Isola & Bacq, 1946) were recorded isotonically. Injections were made through a special T-shaped cannula in the carotid artery. The two short horizontal arms were so shaped that they could be tied into the central and peripheral ends of the artery. The third arm was expanded to form a small reservoir containing air and closed by a rubber cap through which injections were made. Heparin (500 units per kg.) was injected intravenously. The cat's spleen was perfused *in situ* with blood from the carotid artery and records were taken of the splenic volume and the perfusion pressure. Plasma was also tested directly for histamine by its action on isolated guinea-pig's ileum suspended in 2 ml. of atropinized Tyrode solution.

Drugs used:

(1) L-adrenaline;
(2) L-noradrenaline HCl;
(3) DL-α-ethylnoradrenaline HCl $(HO)_2C_6H_3.CHOH.CH(C_2H_5)NH_2$ HCl;
(4) DL-*N*-ethylnoradrenaline HCl $(HO)_2C_6H_3CHOH.CH_2.NH.C_2H_5$ HCl;
(5) DL-*N*-isopropylnoradrenaline HCl;
(6) DL-corbasil HCl $(HO)_2C_6H_3.CHOH.CH(CH_3)NH_2$ HCl;
(7) epinine HCl $(HO)_2C_6H_3CH_2CH_2NH.CH_3$;
(8) adrenalone $(HO)_2C_6H_3.CO.CH_2NH.CH_3$;
(9) hydroxytyramine $(HO)_2C_6H_3CH_2CH_2NH_2$;
(10) tyramine HCl, histamine acid phosphate, dihydroergotamine methanesulphonate, cocaine HCl.

All quantities are expressed in terms of the base.

RESULTS

Splenic blood flow. Stimulation of the splenic nerves for periods of 1 min. caused an initial rapid increase of flow, followed by either a return to the initial rate, a slowing, or a complete cessation of flow. The latter was the most common. Coincidentally with the increase of flow a rise of the general blood pressure occurred, largely due to the increase in circulating blood volume. Consequently it was not possible to determine the effect of splenic sympathin on the blood pressure in the whole animal.

Assays. Parallel quantitative assays by different methods were carried out on the plasma obtained in each experiment. Comparisons with different sympathomimetic amines and other possible active substances were made. Most of the assays have been complicated by the presence of unknown interfering substances in the plasma. These problems are discussed elsewhere (Gaddum *et al.* 1949). Tests have been applied to twenty-nine samples of plasma collected from the splenic vein of thirteen cats after stimulation of the nerves. Tests on the rat's colon showed that in eleven out of thirteen experiments samples collected after stimulation of the nerves had an adrenaline-like effect as compared with that of the arterial sample or of a control sample collected before stimulation. Similar evidence that the appearance of activity followed stimulation of the nerves was obtained with the perfused rabbit's ear, the perfused spleen, the nictitating membrane and lower lid, and in some cases with the rat's uterus (Table 1). The main substance responsible for this effect was certainly not adrenaline. This was clearly proved in every case by the fact

that the plasma had little or no effect on the rat's uterus. If adrenaline had been present in sufficient quantity to account for the effect on the colon, it would have had a large effect on the uterus. Actually seventeen out of the twenty-six samples had the same effect on the uterus as control samples. The remaining samples had a small extra effect, the significance of which is discussed later.

TABLE 1. The sympathin concentration in the post-stimulus samples of plasma in terms of adrenaline and noradrenaline (mμg. per ml.)

N.M., nictitating membrane; L.Lid., lower lid.

	Adrenaline					Noradrenaline				
	Uterus	Colon	Ear	Spleen	N.M. and L.Lid.					
Exp.	1	2	3	4	5	1	2	3	4	5
2 A	> 2·5 < 5	— —	>300 <600	— —	>100 <400	> 250 < 500	— —	> 600 <1200	— —	>100 <400
2 B	< 5 —	> 100 < 350	— —	— —	— 60	— < 500	> 50 <200	— —	— —	— 60
3 A IV	>10 <20	> 150 < 750	— —	— —	— —	> 500 <1000	>100 <500	— —	— —	— —
3 A V	<20	750	—	—	—	<1000	500	—	—	—
3 A VI	<20	150	—	—	—	<1000	100	—	—	—
3 B III	<20 —	> 150 < 750	— —	— —	— —	— <1000	>100 <500	— —	— —	— —
3 C III	<20 —	> 75 < 150	— —	— —	— —	— <1000	> 50 <100	— —	— —	— —
4 A II	<20	100	—	—	< 60	<4000	50	—	—	< 60
4 A III	<20	200	—	—	80	<4000	100	—	—	80
4 B II	<20 —	> 100 < 200	— —	— —	> 40 < 60	<4000 —	> 50 <100	— —	— —	> 40 < 60
6	< 5 —	> 200 < 600	>200 <350	>300 <600	— —	— < 500	>100 <300	> 250 < 500	>300 <600	— —
7	<10	100	< 60	—	—	<1000	100	< 200	—	—
9 A II	< 5	400	30	—	—	<1500	100	75	—	—
9 A III	> 5 <25	1600 —	>150 <250	— —	— —	— 5000	— 400	> 300 < 500	— —	— —
10	< 5	100	—	—	—	< 500	50	—	—	—
11 A II	<10	400	<150	—	—	<1000	80	< 300	—	—
11 A III	<10 —	> 400 <1000	<150 —	— —	— —	<1000 —	> 80 <200	< 300 —	— —	— —
11 B II	<10	250	<150	—	—	<1000	50	< 300	—	—
12 II	2·5	400	—	<500	—	170	200	—	<500	—
12 IV	<2·5 —	> 100 < 200	— —	— <500	— —	— < 125	> 50 <100	— —	— <500	— —
14 II	10	130	—	—	<300	1000	90	—	—	<200
14 III	<10	90	—	—	<300	<1000	65	—	—	<200
14 VI	< 5	75	—	—	—	< 500	50	—	—	—
15 IV	10	130	—	—	<500	1000	100	—	—	<500
15 V	>10 <50	400 —	— —	— —	<500 —	>1000 <5000	300 —	— —	— —	<500 —
15 VI	10	250	—	—	<500	1000	200	—	—	<500

Estimations of the adrenaline equivalent by means of the rabbit's ear gave results significantly greater than those on the rat's uterus (Exps. 2, 6 and 9), and significantly less than those on the rat's colon (Exps. 7, 9 and 11). Estimations with the lower lid and nictitating membrane gave results significantly greater than those on the uterus (Exps. 2 and 4), and less than those on the colon (Exps. 2 and 4). An estimation with the perfused spleen gave an adrenaline equivalent significantly greater than on the uterus (Exp. 6). There can be no doubt therefore that the active substance is not adrenaline.

Similar, but less complete, evidence was obtained which excludes from consideration all the other substances tested except noradrenaline, and perhaps ethylnoradrenaline. The most clearly established property of the active plasma was a very high adrenaline equivalent on the colon as compared with the uterus. This property was rare among the substances previously tested (Gaddum *et al.* 1949). It is not possessed by hydroxytyramine, epinine, adrenalone, corbasil, *N*-ethylnoradrenaline, *N*-isopropylnoradrenaline, or tyramine, and these substances could be excluded from consideration on this ground alone, but a number of direct comparisons have also been made. Corbasil was compared with an active plasma in Exp. 6. The concentration indicated by the rabbit's

TABLE 2. The sympathin concentration in the post-stimulus samples of plasma in terms of various amines (mμg. per ml.)

Exp.	Drug	Membrane	Colon	Uterus	Ear	Spleen
6	Histamine	—	>10,000	—	> 2,500	*
		—	<30,000	—	< 5,000	
6	Corbasil	—	> 1,000	—	>250,000	*
		—	< 5,000	—	<500,000	
7	Epinine	—	10,000	—	< 1,200	—
11 A II	Adrenalone	—	8,000	<250	< 19,500	—
14 II	α-Ethylnoradrenaline	<1500	90	500	—	—

* Vaso-dilatation.

ear was relatively very much higher than the result on the rat's colon, while on the perfused cat's spleen corbasil was a vaso-dilator. On these counts it could be excluded from consideration. Epinine was compared in Exp. 7, and the concentration indicated by the colon was relatively much higher than that compatible with the findings on the rabbit's ear. Therefore this substance was not further considered. Adrenalone was compared in Exps. 11 and 12 of which the results in Exp. 11 are representative. While the concentration as indicated by the colon was compatible with the result on the rabbit's ear, the effect on the uterus was clearly incompatible, excluding adrenalone as the main sympathin. The only other substances known to have approximately the right properties when tested on the rat's uterus and colon are α-ethylnoradrenaline, ephedrine and histamine. α-Ethylnoradrenaline can probably be excluded because its noradrenaline equivalent on the rabbit's ear and the nictitating

membrane is about 1/10 of that on the colon (Gaddum *et al.* 1949). Direct comparison was made between an active plasma and this substance, and the rat's uterus indicated a significantly higher amine content than the colon. This result will be discussed later, together with other similar results.

Ephedrine can be excluded because it has no definite constrictor effect on the rabbit's ear and has a prolonged action on the nictitating membrane, unlike the short duration effects of adrenaline and the active plasmas. The concentrations would have to be very high also (*c.* 10^{-6}).

The active substance cannot be histamine because it causes vaso-constriction in the perfused spleen, where histamine causes vaso-dilatation. Direct tests were made on active plasmas using guinea-pig's ileum. It was shown, for example, that the concentration of histamine was less than 10^{-8} when the concentration necessary to account for the effect on the colon would be about 500 times as much.

The substance cannot be an adenosine compound, since it has practically none of the right properties.

The evidence supports the view that the main active substance is noradrenaline, and all the plasmas have been directly compared with this substance (Table 1). The concentrations of noradrenaline estimated by the rat's colon were from 50 to 500 mμg./ml. In most experiments the plasma had no effect on the uterus, and the equivalent amount of noradrenaline would also have had no effect. The result of the experiment with most parallel assays is shown in Fig. 1, and there is fair agreement between the concentrations indicated by the various tests. While the distribution of concentrations is rather wide, the low concentrations of active substance obtained in these experiments imply that most of the test objects were being used at their threshold levels of response, where discrimination is not easy, and up to a 100% error is possible. A significant difference then is one which is greater than this. However, close agreement has been obtained in many experiments (Table 1), and all the other substances considered, except one, were excluded on this basis. In Fig. 1 no value for the assay on the uterus has been given. In this experiment the adrenals were still present, and though the effect shown is slight, all the samples, control and arterial, were active on the uterus. The arterial sample was the most active. This uterus was extremely sensitive to adrenaline, and could detect 0·2 mμg. added to the bath (concentration in the added solution of 10^{-9}). In one other experiment also control samples had this effect on the uterus, but in all subsequent experiments, in which the adrenals were removed, such activity was absent. This activity was, therefore, regarded as due to adrenaline released from the adrenals. However, the tracing in Fig. 1 serves to show the slight effect on the uterus in relation to the other tests. When adrenaline was added to the stimulus sample to give a known concentration of 5 mμg./ml. a difference between it and the control was shown. Therefore the active substance shown

by the other test objects could not be adrenaline in the concentration indicated by them. The stimulus sample would have had a very large effect on the uterus. The adrenals were present in Exps. 2–7, and absent in Exps. 9–15. On the rat's colon in Fig. 1 the control sample caused both a contraction on addition and an increase in the subsequent acetylcholine response. In contrast, the stimulus sample caused no contraction on addition to the bath, and very slightly

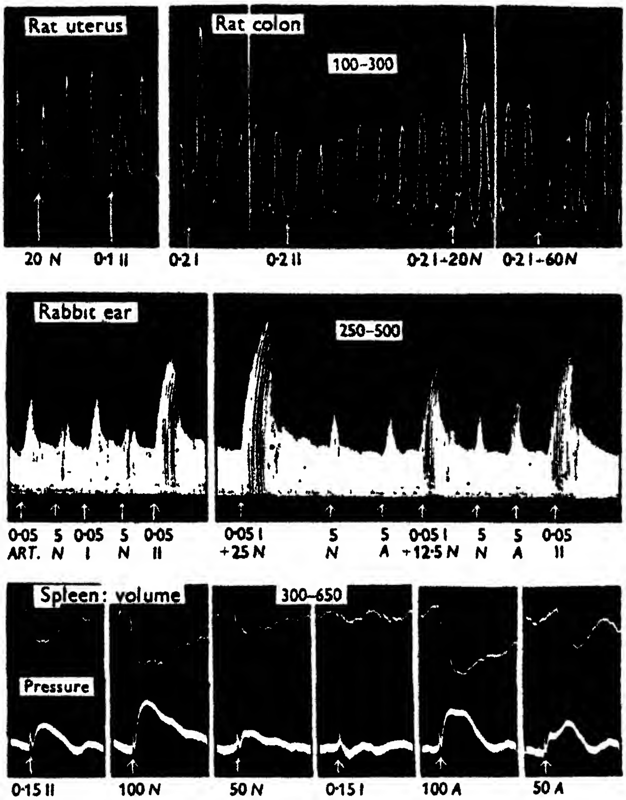


Fig. 1. Parallel assays (Exp. 6). Splenic plasma before (I), and after (II) stimulation of nerves. *ART.*, arterial plasma; *A*, adrenaline; *N*, noradrenaline. Doses in mμg. and ml. Noradrenaline equivalents (mμg. per ml.) above records.

inhibited the subsequent response to acetylcholine, in similar fashion to the control samples to which noradrenaline was added. The use in the later experiments of extra heparin (10 units/ml.) in the collecting tube, to prevent the appearance in the control samples of this undesirable activity, is shown by the assay on the uterus and colon illustrated in Fig. 2. On the rabbit's ear in Fig. 1 both the arterial and control splenic samples caused vaso-constriction, but the stimulus sample had a much larger effect, and comparison with the control plasma to which amine was added could be made. Later it was discovered that

these unknown vaso-constrictors in control and arterial plasma were inhibited by small doses of dihydroergotamine methane-sulphonate injected previously by the arterial cannula, and it was then possible to show that activity remained only in the stimulus sample. This was because the unknown substances were often more sensitive to dihydroergotamine than was adrenaline or noradrenaline. This is illustrated in Fig. 3. The stimulus sample then behaved like a solution of adrenaline or noradrenaline and could, like them, be inhibited by larger doses of dihydroergotamine. As the effects of this drug wore off, the activity

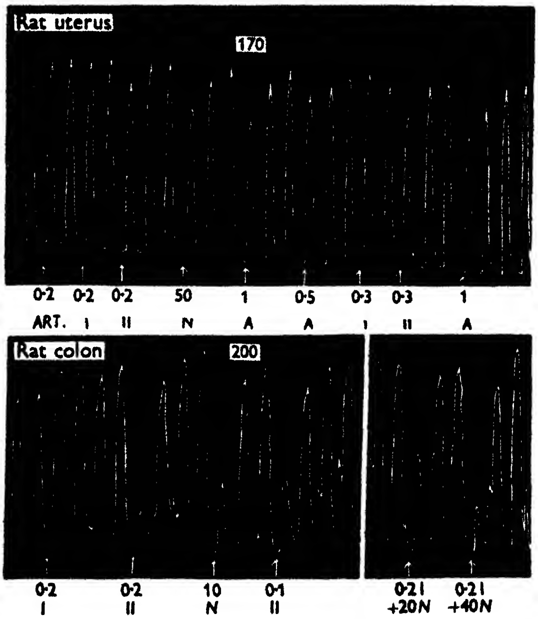


Fig. 2. Parallel assays (Exp. 12, II). Splenic plasma before (I) and after (II) stimulation of nerves. *ART.*, arterial plasma; rat uterus, 170 mμg. per ml. (50 N = 0·3 II); rat colon, 200 mμg. per ml. (0·2 I + 40 N = 0·2 I I).

of the stimulus sample returned at the same rate as that of comparable amounts of adrenaline and noradrenaline. In the case of the perfused spleen in Fig. 1 the control sample had practically no effect, while the stimulus sample caused a decrease in volume and a rise of inflow pressure; similarly, no effect was produced by the control plasma of Exp. 4 on the lower lid (Fig. 4). The response to the plasma collected after stimulation could then be matched against amine solutions, provided plasma by itself did not interfere with the response. The importance of adding the amines for comparison to the control plasma for assay purposes must be emphasized. It is demonstrated by the assay on the rat's colon in Exp. 12 (Fig. 2), where half the amine content is masked on addition to the control plasma. Only where this has been done and there is no

interference with the assay, can saline solutions of the amines be used for comparison.

In two experiments (Exps. 2 and 12), the noradrenaline equivalent on the uterus was not very different from that on the colon. Such a result is shown in Fig. 2. The ratio of activity adrenaline to noradrenaline in these uteri was lower than usual so that noradrenaline was more active in this test than usual. In three other experiments (Exps. 9, 14 and 15) the noradrenaline equivalent on the uterus was significantly higher than on the colon. In these experiments noradrenaline cannot have been the only substance liberated. The results can be explained on the theory that stimulation liberated a small quantity of

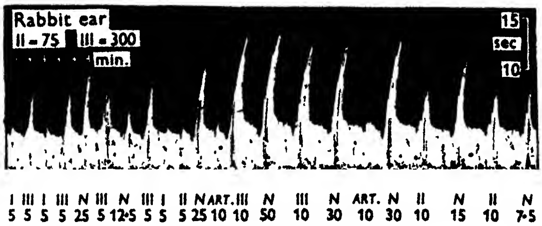


Fig. 3. Outflow from rabbit's ear (Exp. 9A) after dihydroergotamine (5 mμg.). Splenic plasma before (I) and after (II and III) stimulation of nerves. *ART.*, arterial plasma; *N*, noradrenaline. Doses in mμg. and 1/100 ml. Noradrenaline equivalents (above) in mμg. per ml.

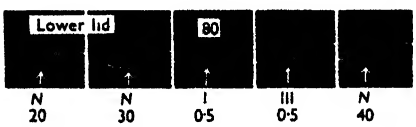


Fig. 4. Cat. Lower lid. Intracarotid injections (Exp. 4). Splenic plasma before (I) and after (III) stimulation of nerves. *N*, noradrenaline (dose in mμg.). Noradrenaline equivalent (80 mμg./ml.).

adrenaline in addition to noradrenaline. The quantities involved are too small to affect the result of any test in which adrenaline and noradrenaline have about equal effects, as they have in the other tests used here. The ratios in the three experiments of the amount of noradrenaline to adrenaline present would be 40, 10 and 10 respectively. This evidence was obtained in the later experiments when the technique was best and the adrenals had been removed. The arterial samples were inactive. It is not unlikely that small amounts of adrenaline are always liberated in addition to noradrenaline. Here the comparison noted above of an active plasma with α-ethylnoradrenaline must be reconsidered. It can be seen that the values on the uterus and the colon in Exp. 14 (Table 2), are like those for noradrenaline in the same experiment and in the other two experiments just considered (Exps. 9 and 15, Table 1), and the result could similarly be explained on the theory of a mixture with adrenaline. The reasons

for believing that this substance is not the main sympathin have already been given.

Estimations of the noradrenaline equivalent on the colon have agreed with those on the rabbit's ear (Exps. 6 and 9), the perfused cat's spleen (Exp. 6), and the nictitating membrane (Exps. 2 and 4). In various other experiments the concentration was too low to be detected by these other tests, but the results were compatible with the theory that the main active substance was noradrenaline.

The circulation through the spleen usually slows or stops after stimulation of the nerves, and it was possible that this was a causal factor in the release of active substances, especially as in some experiments most of the activity was contained in the blood collected during the period of slow flow. Therefore in Exp. 15 the splenic artery was clamped for 2 min. and the splenic venous blood collected after release of the clamp. This sample had no detectable activity when tested on the rat's uterus and colon, and behaved like the control sample. Subsequent stimulation of the nerves released sympathin (Table 1). Further evidence that ischaemia was not the explanation of the appearance of sympathin lay in the fact that blood collected during the period of rapid flow at the start of stimulation in many experiments contained as much or more sympathin.

Usually the sympathin content of plasma collected after a second stimulation of the nerves later in the same experiment was lower than that in the first sample. In two experiments (Exps. 5 and 13), despite sensitive test objects, no sympathin could be detected at any time, even though the spleen contracted well on stimulation of the nerves. The reason for this may be that the sympathin usually detected is the excess over that required to cause a response in the sensitive cells in the spleen, and which passes into the blood. Initially in some, or in the later phases of other experiments, while sufficient is produced to evoke a response in the sensitive cells, insufficient amounts may escape into the blood to be detected by the tests available.

DISCUSSION

The evidence for the liberation of small quantities of adrenaline with the noradrenaline supports the views of Bacq & Fischer (1947), who obtained evidence of such a mixture in extracts of the splenic nerves of the horse and cow. According to Blaschko (1942) noradrenaline is the probable precursor of adrenaline in the body, but it is also possible that the noradrenaline detected in the present experiments was formed by the demethylation of adrenaline as suggested by Bacq & Fischer, or that these substances were present as an equilibrium mixture.

SUMMARY

1. Stimulation of the splenic nerves of cats caused the appearance in the plasma of pharmacologically active substances, which caused contraction of the nictitating membrane or lower lid of cats, vaso-constriction in perfused rabbits' ears and cats' spleens, inhibition of rats' colons and occasionally of rats' uteri.

2. Parallel quantitative assays on the plasma showed that the main active substance was not adrenaline, dihydroxynorephedrine, epinine, adrenalone or histamine. All the evidence supports the view that the substance is noradrenaline.

3. Smaller amounts of adrenaline were sometimes also liberated.

I am indebted to Prof. J. H. Gaddum for his suggestions and his help in the presentation of this work. I am grateful to Dr M. Vogt for performing some of the assays on the rat uterus.

Samples of the amines 2–5 were provided by Messrs Sterling-Winthrop through the kindness of Dr M. L. Tainter.

This work was done during the tenure of a studentship under the Medical Research Council.

REFERENCES

Bacq, Z. M. & Fischer, P. (1947). *Arch. int. Physiol.* **55**, 73.

Bacq, Z. M. & Fredericq, H. (1935). *Arch. int. Physiol.* **41**, 334.

Barcroft, J., Nisimaru, Y. & Puri, S. R. (1932). *J. Physiol.* **74**, 321.

Blaschko, H. (1942). *J. Physiol.* **101**, 337.

Bülbring, E. (1944). *J. Physiol.* **103**, 55.

Cannon, W. B. & Rosenblueth, A. (1937). *Autonomic Neuroeffector Systems.* New York: Macmillan Co.

Euler, U. S. von (1946*a*). *Acta physiol. Scand.* **11**, 168.

Euler, U. S. von (1946*b*). *Acta physiol. Scand.* **12**, 46.

Gaddum, J. H. & Goodwin, L. G. (1947). *J. Physiol.* **105**, 357.

Gaddum, J. H. & Kwiatkowski, H. (1939). *J. Physiol.* **96**. 385.

Gaddum, J. H., Jang, C. S. & Kwiatkowski, H. (1939). *J. Physiol.* **96**, 104.

Gaddum, J. H., Peart, W. S. & Vogt, M. (1949). *J. Physiol.* **108**, 467.

Greer, C. M., Pinkston, J. O., Baxter, J. H. & Brannon, E. S. (1938). *J. Pharmacol.* **62**, 189.

Holton, P. (1949). *Nature, Lond.*, **163**, 217.

Isola, W. & Bacq, Z. M. (1946). *Arch. int. Physiol.* **54**, 30.

de Jalon, P. G., Bayo, J. B. & de Jalon, M. G. (1945). *Farmacoterapia Actual*, **2**, 313.

Loewi, O. (1936). *Pflüg. Arch. ges. Physiol.* **237**, 504.

Schäfer, E. A. & Moore, B. (1896). *J. Physiol.* **20**, 1.

J. Physiol. (1949) 108, 502–507 547.457.1:612.817.1

THE DEPENDENCE OF NEUROMUSCULAR TRANSMISSION ON GLUCOSE

By I. HAJDU and R. J. S. McDOWALL

From King's College, University of London

(*Received* 13 *October* 1948)

In a previous paper (McDowall, Shafei & Miechowsky, 1949) it was shown that 0·2% glucose depressed the activity of the rat diaphragm, and evidence was put forward that the depression was due to a decreased synthesis of acetylcholine. It was also observed that prolonged deprivation of glucose resulted in failure of the preparation to stimulation. It was not possible to locate the site of the failure because when the nerve failed the muscle ceased to respond also. In the present experiments, however, short-duration stimuli were used to reduce the possibility of repetitive responses and such stimuli we now know cannot stimulate muscle. It has now been found that the failure to respond is due to a neuromuscular block and that the muscle will respond for a considerably longer period to direct stimulation if stimuli of adequate duration and strength are employed.

METHOD

The method, which is a slight modification of that used by Bülbring (1946), has been that already described (McDowall *et al.* 1949) with two further modifications. In most experiments the muscle and its nerve were totally immersed in the bath. In this way it was found that in stimulating the muscle directly there was less disturbance from bubbles at the surface of the fluid although stronger currents were needed. Controls were also done in which the condition of the nerve at the point of stimulation was kept constant. Generally Krebs' solution (Krebs, 1932) was used, but this was not essential. This solution has the great advantage that when aerated with 95% oxygen and 5% carbon dioxide it does not undergo any change in pH and the preparation is apparently much more responsive than in Tyrode solution. It has the composition:

NaCl	0·692%	$NaHCO_3$	0·21%
KCl	0·0354%	KH_2PO_4	0·162%
CaCl	0·0282%	$MgSO_4.7H_2O$	0·0294%

RESULTS

If the preparation is deprived of glucose, there is after a variable time an augmentation of contraction followed by a depression. The onset of depression usually takes from 1½ to 2½ hr., but there is much variation in different preparations. Some acted for 7 hr. without glucose. A study of the effect of glucose

lack in different variations in different solutions has shown that the time of onset of depression is a function of the amount of potassium in the solution. Thus using Krebs' solution containing 0·035% potassium chloride, withdrawal of glucose required 2½ hr. while using a solution containing 0·07% potassium chloride caused depression to appear in less than 15 min. Changing the fluid in the bath and increasing the frequency of stimulation to 5/min. shortens the period necessary to produce the block, but any increase beyond this rate is liable to produce a contracture. Illustrations of the augmentation have already been given (McDowall, Shafei & Miechowsky, 1949). Fig. 1 shows the later depression, during which the nerve and the muscle were stimulated alternately with groups of six stimuli; the nerve at 3 V. at 150 μsec. duration and the muscle through its tendon with stimuli of 20 V. and 2800 μsec. duration. It is

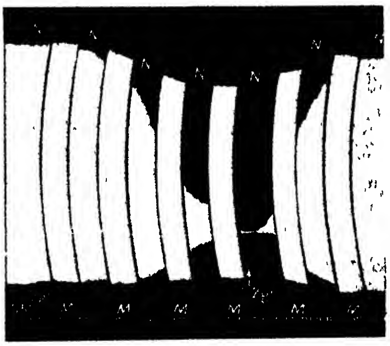


Fig. 1. Nerve (N) and muscle (M) stimulated in alternate groups, the nerve with a current of 3 V. of 150 μsec. duration, the muscle with 20 V. at 2800 μsec. duration. At the arrow a piece of tracing occupying 1 hr. was removed to facilitate reproduction. The indirect response is seen to fail completely while the muscle continues to respond. At 0·2 gl., glucose was added to give a concentration of 0·2% in the bath.

seen that after failure of the nerve the muscle responds well, although a little less than at first, presumably because the response to direct stimulation of the muscle before the block was really due in part to stimulation of the nerve elements within it, as emphasized by Rushton (1930). Observations on the chronaxie of the muscle stimulated directly in a bath empty of fluid showed that the excitability of the muscle had decreased from 250 μsec. before the block to about 1450 μsec. after the block, that is, to the value which is found in curarized or denervated diaphragm.

That the failure of the nervous stimulus is not due to fatigue from repetition of the stimulation is shown by the immediate recovery of the response when glucose was added, the addition of sufficient to bring the concentration to 0·01% being enough for this. If, however, the deprivation of glucose is prolonged after the block occurs, there is a corresponding delay in or even failure

of recovery, as if the mechanism for glucose usage was damaged. This is particularly true if Tyrode solution aerated with oxygen only is used. Once the block has been produced and removed by the addition of glucose, it can be produced again in a few minutes by withdrawal of the glucose.

Substitutes for glucose. A number of substances were tried as possible sub-

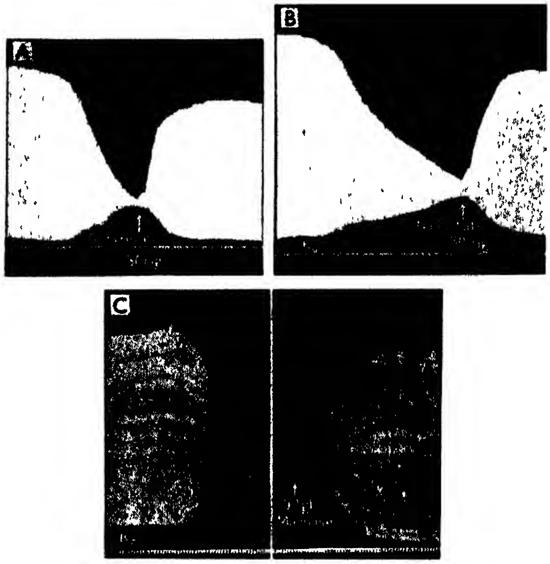


Fig. 2. The nerve was stimulated as before till the block occurred. At the arrows in A, B and C, sodium pyruvate (0·06%), sodium lactate (0·06%) and mannose (2%) respectively were added to the percentages in the bath indicated.

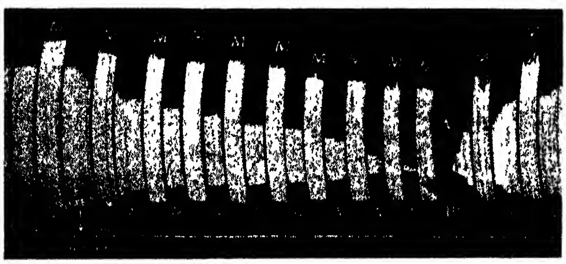


Fig. 3. The nerve (N) and muscle (M) were stimulated as in Fig. 1. At the arrow, 200 mg. phloridzin was added and at W it was replaced by normal Krebs' solution.

stitutes for glucose. Glycine, alanine, glycerol, sucrose, lactose, galactose, aceto-acetate, succinic acid, fructose, malic acid, fumaric acid, citric acid, and aspartic acid were found to be ineffective but the glucose was successfully replaced by mannose (Fig. 2), pyruvic acid (Fig. 2) and lactic acid (Fig. 2) in concentrations of equivalent calorific value. The effectiveness of these substances appeared to be in the order given; mannose brought about a more

immediate recovery than glucose itself. The cause of the differences will be made the subject of subsequent investigation.

Relation to oxygen supply. If the oxygen supply to the preparation is reduced the block due to lack of glucose appears more rapidly. An attempt was made to determine how far the recovery which occurred when glucose and other substances were added was an anaerobic or aerobic phenomenon. The results were complicated by the effect of oxygen lack itself which also causes a neuromuscular block.

Relation to carbon dioxide. Recovery of the neuromuscular transmission occurred appreciably more rapidly if the preparation was aerated with carbon dioxide as well as oxygen. Without carbon dioxide the recovery was never complete.

Effects of iodo-acetate and phloridzin. A considerable number of experiments were carried out in the hope that a block could be produced as a result of a failure of phosphorylation. No concentration of iodoacetate could be found which could produce a block without at the same time poisoning the muscle, nor was the effect reversible. The action of phloridzin, on the other hand, is easily demonstrated. This substance produces an easily reversible neuromuscular block (Fig. 3).

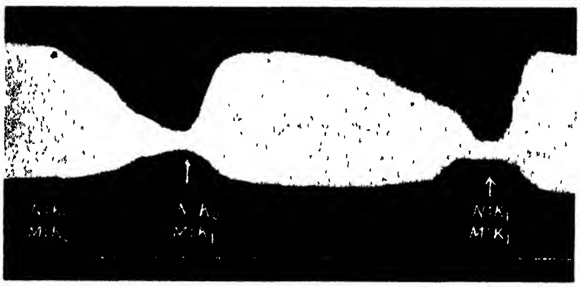


Fig. 4. The nerve only was stimulated till a block occurred. Recovery took place when 0·2% glucose was added to the second chamber containing the muscle but a second block occurred 90 min. later. This was relieved by putting glucose in the first chamber containing the electrodes.

Effect of eserine. After a few minutes of neuromuscular block, the potentiating effect of eserine which is normally seen with doses as small as one in a thousand million, is no longer obtainable. 'Eserine twitches' are also absent. Immediately after the block occurs a small effect may be seen, presumably because the apparent mechanical block is followed by a short period during which the muscle contracts isometrically because of the weight of the recording lever. If, however, acetylcholine is added to the bath as well as eserine no contracture is produced like that seen in denervated muscle, a fact which suggests that the glucose lack acts on the nerve side of the sole plate.

The exact location of the block. The absence of the action of eserine after the block has been produced suggested that the absence of glucose prevented the synthesis of acetylcholine. That a block was produced at the nerve ending was clearly shown by placing the preparation in a double chamber of perspex in which the nerve passed from one section to the other through a hole in the partition. When the block was produced it was at once relieved by adding glucose to the chamber containing the muscle and nerve endings, although the upper part of the nerve on which the stimulating electrodes lay was still subjected to glucose lack About $1\frac{1}{2}$ hr. later, however, a second block occurred which was apparently in the nerve itself for it was relieved by adding glucose to the first chamber. It is thus seen that two varieties of block occur, one at the nerve ending and a later one in the nerve (Fig. 4).

DISCUSSION

The results show that when the rat diaphragm is deprived of glucose two varieties of block occur, the first obscuring the second. The latter would seem to be due to simple failure of the nerve elements, but the first can be related to the neuromuscular junction. Since it has been shown by Feldberg (1945) that brain slices do not synthesize acetylcholine if deprived of glucose and by Kahlson & MacIntosh (1939) that the perfused superior cervical ganglion fails to synthesize acetylcholine in the absence of glucose, it is suggested that the early block is due to failure of the synthesis of acetylcholine in the region of the nerve ending. This is supported by the absence of the action of eserine after glucose block, the large change in chronaxie and the absence of any effect on nerve within the period of the experiment. The exact nature of the subsequent block which is the result of more prolonged exposure of the nerve to glucose lack was further investigated to determine if it also could be related to acetylcholine but without success. If it had been, it might have been expected that the onset of the block due to glucose lack could be delayed temporarily by eserine, but this did not occur.

The facts given suggest that the mechanism of neuromuscular transmission may be linked to the energy mechanism in the muscle, and the muscle may thereby be protected against complete exhaustion of the carbohydrate stores necessary for its resting metabolism. It is probable that in the oxidation of pyruvic acid produced by the breakdown of glucose, thiamine, which von Muralt (1946) has suggested is connected with humoral transmission, plays a part.

By the use of the block produced by glucose lack it becomes possible for the first time to study the pure muscle preparation without the use of curare or denervation, and the results already obtained in relation to chronaxie completely support the view of Rushton (1930) and further negatives Lapicque's theory of isochronism. This becomes the easier because the difference between

the chronaxie of nerve and that of muscle is much greater in the mammal than in the amphibian on which most observations on chronaxie have been made, and the results suggest that differences which have been found in the chronaxies of different muscles are really due to differences in nerve chronaxies or possibly the amounts of acetylcholine available at nerve endings. Indeed the results generally suggest that the effect of any given stimulus must depend on the amount of acetylcholine available to be liberated, a suggestion already put forward by McDowall, Shafei & Miechowsky (1949) who found that excessive amounts of glucose depressed acetylcholine synthesis.

By examining the substitutes for glucose it is possible to study carbohydrate metabolism at least in the region of the nerve ending. Such studies have failed to demonstrate that malic, fumaric or citric acids can replace the glucose which, according to the suggestion of Krebs, Salvin & Johnson (1938), might be expected to do if these substances were normal breakdown products between pyruvic acid and carbon dioxide. The enhancement of the effect of glucose by oxygen lack seems to us to suggest that the neuromuscular transmission involves oxidative processes.

SUMMARY

If the rat diaphragm is deprived of glucose after a variable period neuromuscular transmission ceases, but the muscle continues to respond to direct stimulation of suitable duration. Evidence is put forward that the block is due to failure of acetylcholine synthesis at the nerve endings. It can also be shown that appreciably later a block due to an action on nerve occurs.

The blocked preparation may be used for the study of the properties of muscle and of some aspects of carbohydrate metabolism.

In the completion of this paper we are indebted to a number of our colleagues, Dr Miechowsky, G. J. Allum, J. S. Cornes who, in the course of other work, elaborated and confirmed several points.

REFERENCES

Bülbring, E. (1946). *Brit. J. Pharm.* **1**, 1.
Feldberg, W. (1945). *J. Physiol.* **103**, 367.
Kahlson, G. & MacIntosh, F. C. (1939). *J. Physiol.* **96**, 277.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Krebs, H. A., Salvin, A. & Johnson, E. W. (1938). *Biochem. J.* **32** 113.
McDowall, R. J. S., Shafei, A. Z. & Miechowsky, W. (1949). *J. Physiol.* **108**, 24.
MacIntosh, F. C. (1938). *J. Physiol.* **93**, 46*P*.
von Muralt, A. (1946). *Die signal übermittlung im Nerven*, p. 293.
Rushton, W. A. H. (1930). *J. Physiol.* **70**, 334.

J. Physiol. (1949) 108, 508–524 547.435.292:612.174

ACTION OF ACETYLCHOLINE ON RABBIT AURICLES IN RELATION TO ACETYLCHOLINE SYNTHESIS

By EDITH BÜLBRING AND J. H. BURN

From the Department of Pharmacology, University of Oxford

(*Received* 25 *December* 1948)

In a recent paper (Burn & Vane, 1949) the hypothesis has been discussed that the action of acetylcholine on the heart is not restricted to that of a humoral transmitter of vagal impulses, but that acetylcholine is present in heart muscle playing an important part in the processes responsible for its spontaneous contractions. This discussion arose from observations showing that when the isolated auricles of the rabbit heart are exposed to the action of proguanil (paludrine) for a sufficient time, their spontaneous contractions cease, and can then be restarted by the addition of acetylcholine to the bath. Further additions of acetylcholine serve to increase the amplitude and rate of the contractions. When the contractions have continued for some time, the normal inhibitory effect of acetylcholine returns.

To test the hypothesis by a method which avoids the use of the substance paludrine, experiments have now been performed on isolated auricles allowed to beat for many hours until they stopped. The addition of acetylcholine under these circumstances has been found to restore spontaneous activity. This phenomenon has been investigated in detail and in its relation to the synthesis of acetylcholine which takes place in auricular tissue.

Experiments on the living tissue

METHOD

Freshly dissected auricles were suspended in an isolated organ bath containing Tyrode solution at 28° C., aerated by a mixture of oxygen and 5% CO_2. When left overnight, the temperature was reduced to that of the room (usually 20–22°) and raised again in the morning to 28° C. During the day, the Tyrode solution was changed about once an hour. Acetylcholine bromide was used for the observations and the doses were expressed in terms of the weight of this salt.

RESULTS

Isolated rabbit auricles were found to beat spontaneously for about 24 hr. The beat continued throughout the day, but sometimes stopped during the night

when the temperature was low. If it had stopped, the raising of the temperature next morning often started it again. Some preparations continued to beat for as long as 48 hr.

The arrest of the beat occurred in different ways. Sometimes the beat became gradually smaller and finally disappeared. Sometimes the beat stopped abruptly. Sometimes the beat became irregular, and periods of arrest alternated with periods of regular activity before the final arrest.

The addition of acetylcholine to the bath was found to restart the contractions in fourteen out of twenty-two experiments. The effect is shown in Fig. 1. In this experiment the beat had ceased for 1 hr.; when acetylcholine was added, it began after a latent period of 2 min. At first the beat was small and infrequent; it gained in rate and amplitude until it reached its maximum in 15 min.; during the next 45 min. it declined to about half the amplitude, at which point the auricles were removed from the bath.

Fig. 1. Isolated auricles of rabbit. Tyrode bath 75 c.c. Temp. 28° C. Auricles suspended in bath on previous day; record begins 1 hr. after the beat stopped. (*a*) Addition of 50 μg. ACh starts the beat again; (*b*) 1 hr. later.

The concentration of acetylcholine required to start the beat varied in different experiments from 1 in 100 million to 1 in 400,000. No connexion was observed between the concentration required and the length of time which had elapsed since the beat stopped. The character of the restarted beat, however, appeared to be related to the duration of the arrest. If the period of arrest was short, 5–15 min., the auricle resumed a regular beat at once; if the period was long, e.g. 1 hr. or more, the auricle began by beating irregularly.

When the acetylcholine was removed by replacing the fluid in the bath with fresh Tyrode, the beat stopped again. An example is given in Fig. 2. At the beginning (Fig. 2*a*) the auricles were still beating spontaneously though with a small amplitude. The addition of 4 μg. ACh depressed and slowed the beat, and the arrest of the beat first occurred on washing out. Two minutes later 4 μg. ACh was again added; there was one beat, and after 30 sec. regular activity started. The acetylcholine was left in the bath for 9 min. and then removed. This produced a short burst of improved activity, followed by arrest.

A repetition of the addition of 4 μg. ACh (Fig. 2*b*) now had much less effect than before; it elicited a series of small contractions which stopped again after

1 min. The addition of 20 μg. started slow but regular activity, and the further addition of 100 μg., after an inhibitory effect during 45 sec., increased both the amplitude and the rate of the contractions. Seven minutes later, double this amount of acetylcholine depressed the beat.

Fig. 2*c* (which follows directly on Fig. 2*b*) demonstrates the removal of the depression by washing, and then the opposed effect of smaller and larger amounts of acetylcholine. The addition of 50 μg. caused stimulation, and the subsequent addition of 100 μg. caused inhibition. At this stage of the experiment the contractions of the auricles were bigger than at the beginning of Fig. 2*a*, and it was difficult to stop them again. However, by changing the fluid in the bath four times during the next 20 min. the beat was arrested, and Fig. 2*d* shows that the dose of 100 μg., which last depressed the beat, now restored it. Activity being resumed, the further addition of 100 μg. caused depression again.

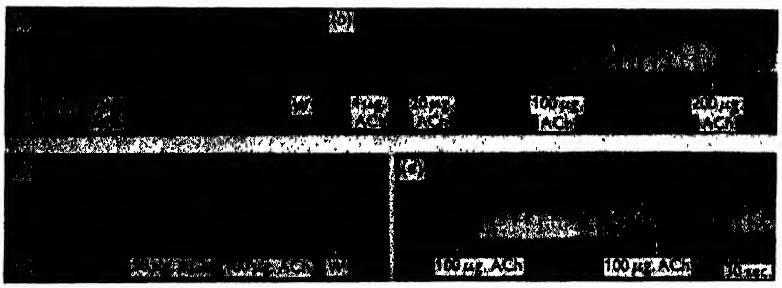


Fig. 2. Rabbit auricles as Fig. 1. (*a*), (*b*) and (*d*) show further examples of the beat restarted by acetylcholine. Note that the same dose of acetylcholine (100 μg.) can stimulate as well as inhibit. See text.

The observation was repeatedly made that a dose of acetylcholine, which had caused inhibition of the normal beat, would start the beat when it had stopped, and that when the beat was resumed the same dose would cause inhibition once more. Further, when first the beat was started it could be stopped by washing out fairly soon, but if activity had been resumed and allowed to continue for some time, then it was difficult to arrest it by washing out.

Before the auricles had stopped their normal beat, no matter how feeble this was, the addition of acetylcholine, however small the dose, was never observed to cause stimulation. When the auricles had been restarted, a further addition of acetylcholine in several experiments caused increase of rate and amplitude (as in Fig. 2*b*, *c*), but as a general rule its effect was again inhibitory. There was, however, a difference. Whereas before the auricles had stopped, the inhibitory effect of small doses of acetylcholine was usually much greater than in freshly prepared auricles; in the restarted auricles, on the other hand, the inhibitory effect was much less.

In Fig. 3, three experiments are illustrated, in the first of which the beat was started by 0·5μg. ACh and in the second by 100μg. ACh (added to a bath of 75 c.c.). In each experiment the effect was prolonged, and the results suggested to us that the duration of the effect was not dependent on the size of the dose, but on some process initiated in the muscle which, once initiated, was self-maintained. At a time when the resumed contractions of the auricle were fully developed they did not stop when the bath fluid containing acetylcholine was replaced by fresh Tyrode. In some other observations the addition of acetyl-

(*a*)

(*b*)

(*c*)

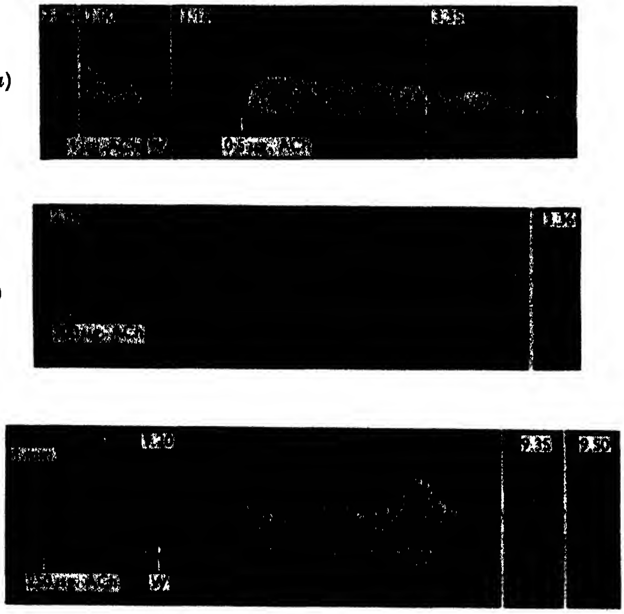


Fig. 3. Rabbit auricles. (*a*) shows (1) initial beat when freshly put up; (2) beat 24 hr. later inhibited by acetylcholine and stopped on washing out the bath; (3) the restart by 0·5μg. ACh; (4) 20 min. later. (*b*) shows another auricle isolated for 26 hr. Stopped for 10 min. before record begins. Restarted by 100μg. ACh; beat 45 min. later. (*c*) shows a third auricle isolated for 31 hr. Stopped 40 min. before record begins. Four previous doses of acetylcholine failed to start the beat, but on washing out the bath the beat began. See text.

choline at first appeared to have no effect, but when it was removed from the bath contractions started. Such an example is shown in Fig. 3*c* in which five successive doses of acetylcholine (1, 5, 25, 125, finally 250μg.) administered during 20 min. did not start the beat. But, on washing out, regular activity began; this we believe was the result of the previous application of acetylcholine.

The experiments so far suggested that when the auricle was exhausted some metabolic process ceased. If the addition of acetylcholine was necessary to initiate this process on which the contractions of the auricle depended, and if

this process continued after the added acetylcholine had been removed, it seemed possible that acetylcholine was synthesized in the heart muscle in order to enable it to contract.

Acetylcholine synthesis

METHOD

We have applied to rabbit auricles the method described by Feldberg & Mann (1946) for estimating acetylcholine synthesis in brain tissue. For this purpose the auricles were converted into an acetone-dried powder, and the amounts of acetylcholine synthesized during 75 min. incubation were expressed in μg. per g. acetone powder. Comline (1946) has recorded that auricles may synthesize up to 90 μg./g./hr. In studying synthesis by brain tissue, Feldberg & Mann found that three factors greatly increased the activity, adenosine triphosphate, citric acid, and a third substance prepared by extracting the acetone powder of brain with boiling saline. This third substance was called 'activator'. The importance of adenosine triphosphate was first pointed out by Nachmansohn & Machado (1943). We incubated our preparations accordingly in the following way for 75 min. To 50 mg. acetone-dried auricle powder suspended in 2 c.c. activator were added:

	c.c.
KCl, 6%	0·1
NaF, 2%	0·1
Choline chloride, 3%	0·1
Eserine sulphate, 0·5%	0·1
Phosphate buffer, M/15	0·3
$MgCl_2$, 4%	0·1
Sodium citrate, 15%	0·1
ATP-P, 0·4 mg. in	0·4
Cysteine, 4·5 mg. in	0·2
Saline	1·0
Total	4·5

Alongside each preparation a control was incubated containing everything except the auricle powder. The estimations of acetylcholine were carried out on the frog rectus muscle, using as standard solutions varying concentrations of acetylcholine made up in the activator control which was diluted to the same extent as the preparation with which it was compared. A detailed example is given later. A few of the later experiments were carried out without converting the auricle tissue into an acetone-dried powder, but grinding the fresh tissue with sand in activator, and after 5 min. centrifugation taking the supernatant fluid for incubation. The amounts of activator and of the other ingredients were calculated after it had been established that one part of acetone-dried powder represented seven parts of fresh tissue. The results obtained with acetone-dried powder and with supernatant fluid from fresh tissue were alike.

RESULTS

Synthesis in relation to activity. Estimations were first made of the synthesizing power of powders from: (*a*) freshly excised auricles, (*b*) auricles which had stopped beating at the end of 20–25 hr., (*c*) auricles which, after stopping, had been started by the addition of acetylcholine.

We made thirty-two observations on fresh auricles and found that the amount synthesized varied from 20 to 75 μg. ACh/g./powder/75 min., the mean figure being $40{\cdot}2 \pm 12{\cdot}7$. The mean figure for stopped auricles was $14{\cdot}9 \pm 10{\cdot}6$. We believe that the wide variation is partly due to variation in the activity of the

ATP preparations used. An investigation of the reason for this variation is in progress, jointly with Dr L. A. Stocken of the Department of Biochemistry, Oxford, to whom we wish to express our thanks for supplying us most generously with the ATP used in this research. In comparing auricles of different kinds, fresh, stopped and restarted, we therefore relied on comparisons between the synthesizing power of each kind on the same day, employing the same reagents. Such results are given in Table 1, in which it is seen that in each experiment the value

TABLE 1. Acetylcholine synthesis by rabbit auricles during 75 min. incubation

			Restarted		Failed to restart	
Exp.	Fresh, synthesized (μg./g.)	Stopped, synthesized (μg./g.)	Synthesized (μg./g.)	ACh added to 75 c.c. bath (μg.)	Synthesized (μg./g.)	ACh added to 75 c.c. bath (μg.)
1	75	<5	40*	50	—	—
2	40	—	35†	100	—	—
3	55	8	—	—	5	100
4	40	8	32	10	5	100
					<5	100
					8	100
5	47	12	40	30	—	—
		18	38	30	—	—

* Determination made on auricle used in Fig. 1.
† Determination made on auricle used in Fig. 3*b*.

for the stopped auricle was much less than that for the fresh auricle, while the value for the restarted auricle approached that of the fresh auricle. The restarted auricles were taken for the determination of synthesizing power 45–60 min. after restarting. The mean figures in the five experiments were, for the fresh auricles 51·4, for the stopped auricles 10·2, for the restarted auricles 37·0. In the first experiment shown in Table 1, the stopped and the restarted auricle had both stopped for 60 min. when the former was taken out of the bath for acetylcholine synthesis, while the beat of the latter was started by the addition of 50 μg. ACh and its synthesizing power was determined 1 hr. later. In the fourth experiment the period of arrest was only 8 min. at the end of which the synthesizing power of the stopped auricle was 8 μg./g. while that of the restarted auricle 45 min. later was 32 μg./g. There appeared to be a clear correspondence between the synthesizing power and the functional state of the auricle.

This conclusion was supported by observations on auricles which had stopped but did not start on the addition of acetylcholine to the bath. On the day of the third experiment in Table 1 two auricles had stopped for 45 min. and the synthesizing power of one was then determined and found to be 8 μg./g. To the other, acetylcholine (100 μg./75 c.c.) was added, but it failed to start. One hour later its synthesizing power was determined and found to be 5 μg./g. The fourth experiment, in which the period of arrest was only 8 min., shows the same point.

This evidence eliminates the possibility that the acetylcholine determined after incubation of the powder might not actually be synthesized but might be the acetylcholine added to the bath.

Further, an experiment was carried out in which two auricles, which had stopped after 25 and 26 hr. respectively, were restarted by the addition of acetylcholine (100 μg./75 c.c.). After 45 min. both were converted to powders and the usual ingredients were added to each. At this point, before incubation, the acetylcholine content of one was determined, while the other was incubated as usual. The non-incubated sample contained no acetylcholine, while the other contained an amount indicating a synthesis of 35 μg./g./75 min.

Effect of acetylcholine on synthesis. Two points had now emerged. The first was that auricles which had stopped could be started again by the addition of acetylcholine. The second was that auricles which had stopped had lost most of their power of synthesis, but regained it when the contractions were restarted. These facts suggested that acetylcholine might influence the power of synthesis *in vitro*.

An experiment was therefore performed, part of which is already recorded as the last experiment in Table 1. The powder from the fresh auricle was divided into two equal parts, and to one of these, before incubation, 0·25 μg. ACh was added per 50 mg. powder. The powder from each of the two stopped auricles was also divided into two equal parts, and the same proportion of acetylcholine (0·25 μg./50 mg. powder) was added to one of them. The results are given in Table 2. The figures in Table 2 show that the addition of acetylcholine before incubation diminished the synthesis of acetylcholine by the preparation from the fresh auricle, while it augmented the synthesis by the preparation from the stopped auricle.

TABLE 2. Acetylcholine synthesis in rabbit auricles (μg./g./75 min.)

	Incubated without ACh	0·25 μg./50 mg. ACh added before incubation
Fresh	47	33
Stopped	12	20
Stopped	18	30

The estimation was carried out in the following way. For each sample of powder incubated with acetylcholine a control sample containing the same amount of acetylcholine was incubated alongside. By estimation on the frog rectus, the amount of acetylcholine synthesized was determined in comparison with known amounts of acetylcholine added to this control sample. Having thus obtained one estimate of the amount of acetylcholine synthesized, a second estimate was obtained by comparing the solution containing the powder incubated with acetylcholine with the solution containing the powder incubated without acetylcholine after adding the difference found by the first estimate. Good agreement was obtained.

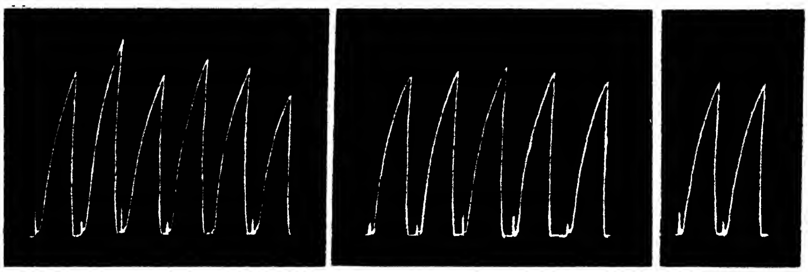
An example is shown in Fig. 4. *A* is the solution containing the powder incubated without acetylcholine. Fig. 4*a* shows that:

2 c.c. *A* is less than 0·2 μg. ACh in 2 c.c. activator control;

2 c.c. *A* is less than 0·18 μg. ACh in 2 c.c. activator control;

2 c.c. *A* is greater than 0·16 μg. ACh in 2 c.c. activator control.

Therefore 2 c.c. $A = 0{\cdot}17\,\mu$g. ACh.



2 A 0·2 μg. 2 A 0·18 μg. 2 A 0·16 μg. (*a*)

0·04 μg. 1 B 0·045 μg. 0·04 μg. 1 B (*b*)

1 A + 0·115 μg. 1 B (*c*)

Fig. 4. Part of an acetylcholine assay on the frog rectus described in detail in the text.

As 2 c.c. *A* contained 4 mg. powder, the amount synthesized per g. powder was

$$\frac{0{\cdot}17}{4} \times 1000 = 43\,\mu\text{g./g./75 min.} \tag{1}$$

B is the solution containing the powder incubated with acetylcholine. The solution contained 2 mg. powder/c.c., and acetylcholine had been added before incubation in the amount of 80 μg./g., or 0·16 μg./2 mg. Fig. 4*b* shows that: 1 c.c. *B* (incubated with 0·16 μg. ACh) was almost equal to 0·04 μg. ACh in 1 c.c. activator control (also incubated with 0·16 μg. ACh per c.c). As 1 c.c. *B* contained 2 mg. powder, the amount synthesized was

$$\frac{0{\cdot}04}{2} \times 1000 = 20\,\mu\text{g./g./75 min.} \tag{2}$$

The total amount of acetylcholine in 1 c.c. *B* was thus

$$0{\cdot}04 + 0{\cdot}16 = 0{\cdot}2\,\mu\text{g. ACh.}$$

Now 2 c.c. *A* contained 0·17 μg. ACh.,

1 c.c. *A* contained 0·085 μg. ACh.

Hence the amount of acetylcholine to be added to 1 c.c. *A* so that 1 c.c. *A* should produce the same effect as 1 c.c. *B* is

$$0{\cdot}2\,\mu\text{g.} - 0{\cdot}85\,\mu\text{g.} = 0{\cdot}115\,\mu\text{g.}$$

This calculation was confirmed as shown in Fig. 4*c*, in which 1 c.c. $A + 0{\cdot}115\,\mu$g. is equivalent to 1 c.c. *B*.

If both portions of the same auricle powder had synthesized the same amount of acetylcholine, i.e. 43 μg./g., then the difference between *A* and *B* would have been 80 μg./g., since this was the amount added to *B* before incubation. But actually to match the contraction of the frog rectus produced by 1 c.c. *B* only 0·115 μg. ACh had to be added to 1 c.c. *A*. An addition of 0·115 μg. to 2 mg. corresponds to an addition of 57 μg./g. Since $80 - 57 = 23\,\mu$g., this confirms the conclusions in (1) and (2) that sample *B* synthesized 23 μg./g. less than sample *A*.

The rate of synthesis. In order to study the effect of acetylcholine upon the rate of synthesis, as well as on the total amount synthesized, two experiments, one on six auricles and one on seven, were carried out. In both the fresh tissue was ground in activator, and, after centrifuging, the supernatant fluid was used for incubation. In each experiment six aliquot portions were taken, three of which were incubated with 1 μg. acetylcholine, and three without. At varying intervals, a pair of samples (one with ACh and one without) was removed for estimation. The results are shown in Fig. 5 and it is seen that the synthesis at

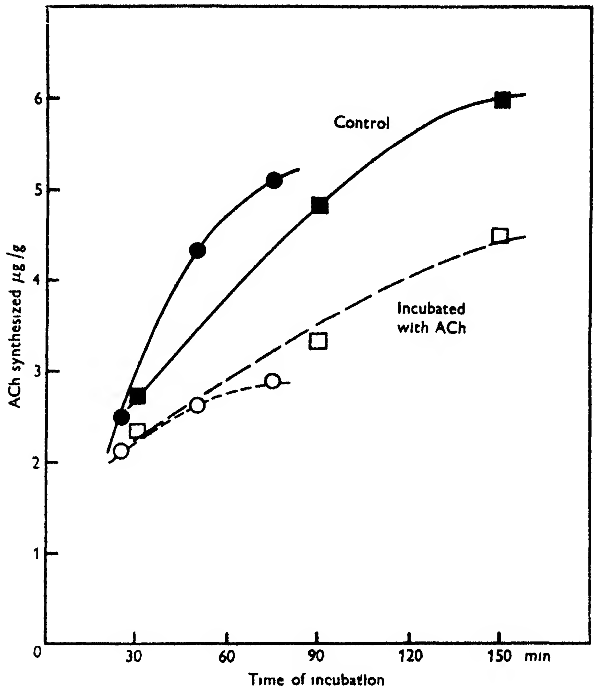


Fig. 5. Two experiments showing the rate of synthesis of acetylcholine by fresh auricle extract (continuous lines), and the slowing of the rate by the addition of acetylcholine (1 μg. to extract of 350 mg. auricle) (broken lines). Ordinates: acetylcholine synthesized (μg. per g. fresh weight). Abscissae: minutes. First experiment shown by squares; second experiment shown by circles.

all points of both experiments is depressed by the addition of acetylcholine. The addition of acetylcholine does not act by allowing synthesis to proceed at a normal rate until a certain total amount is reached, at which point the synthesis stops; but the addition of acetylcholine slows the rate of synthesis at an early stage.

Effect of varying amounts of acetylcholine on fresh auricles. The inhibition by acetylcholine of the synthesis in preparations from fresh auricles shown in Table 2 and Fig. 5 was studied in a series of experiments in which different

amounts of acetylcholine were added to portions of the same powder. For this purpose a sufficient quantity of acetone-dried powder was prepared from the auricles of two, three, or four rabbits for each experiment. The results of fifteen experiments are given in Table 3. In each of these experiments the

TABLE 3. Acetylcholine synthesized by fresh auricles

(μg./g./75 min.)

Incubated without ACh	ACh added per g. acetone powder				
	5 μg.	10 μg.	20 μg.	40 μg.	80 μg.
20	6	5	3	2	—
22	16	12	9	—	—
25	18	19	—	—	0
25	20	20	—	—	6·5
32	—	—	23	—	—
33	—	—	22	—	—
33	—	27	—	22	—
36	—	—	—	18	—
36	—	—	21	—	—
38	—	37	—	—	—
40	—	—	—	25	20
40	—	35	33	31	—
43	—	—	20	—	—
43	—	—	24	27	20
45	33	—	—	—	—
45	40	—	—	—	—
47	33	—	—	—	—

addition of acetylcholine diminished the amount synthesized. The diminution was greater the greater the amount added. In one experiment the synthesis was entirely suppressed by adding 80 μg./g. powder. The mean course of the results is shown in Fig. 6, curve *A*, in which the percentage depression is expressed. The points were obtained by calculating each figure in Table 3 as a percentage of the control figure in the same experiment, and then determining the mean percentage for each amount of acetylcholine added. The curve *A* in Fig. 6 falls to 30% of the initial synthesis; the fall is steepest at first, the addition of 5 μg./g. reducing the synthesis to 70%.

Effect of varying amounts of acetylcholine on stopped auricles. Similar experiments were carried out with preparations made by pooling auricles which had been set up in isolated organ baths the day before and taken down at the end of 21–26 hr. Some of them had stopped beating, but others had not. We have divided the results accordingly. Table 4 gives results of stopped auricles only, while Table 5 gives results of auricles which were taken out of the bath simultaneously but of which only a proportion had stopped.

In Table 4 it is seen that in each experiment the addition of acetylcholine increased the synthesis. In the first experiment the synthesis was doubled with the addition of 5 μg./g., and trebled with the addition of 10 μg./g. With further additions, the increase became less, but the synthesis did not fall below that of the control sample.

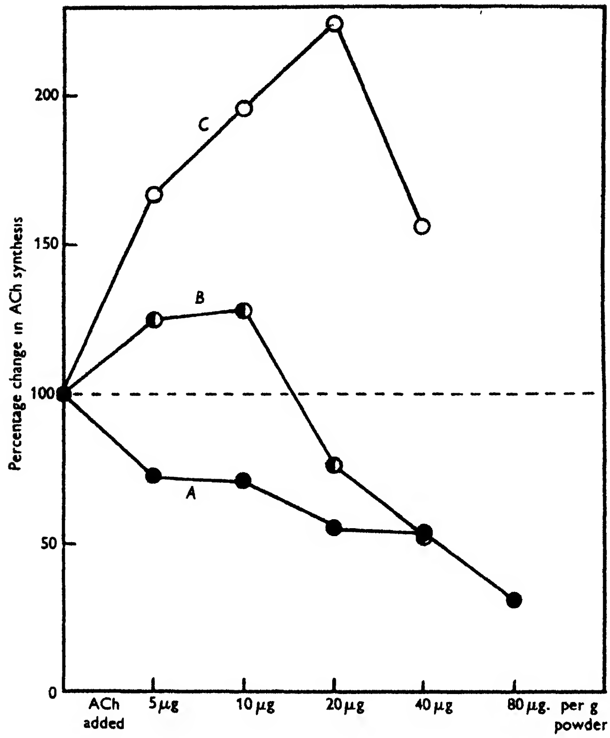


Fig. 6. Effect of acetylcholine on synthesis of acetylcholine by acetone powder prepared from fresh auricles (*A*), stopped auricles (*C*) and auricles not all stopped (*B*). The synthesis in the absence of acetylcholine is taken as 100. The points are calculated from the values in Tables 3–5. Ordinates: percentage change in synthesis. Abscissae: amounts of acetylcholine added per g. powder.

TABLE 4. Acetylcholine synthesized by stopped auricles (µg./g./75 min.)

Incubated without ACh	ACh added per g. acetone powder			
	5 µg.	10 µg.	20 µg.	40 µ
4·5	9	15	13	7
5	—	—	8	—
12	20	—	—	—
18	30	—	—	—
19	—	29	—	—
27	37	—	—	—
29	—	50	—	—
32	—	40	—	—

TABLE 5. Acetylcholine synthesized by auricles not all stopped (µg./g./75 min.)

Proportion of auricles which stopped	Incubated without ACh	ACh added per g. acetone powder			
		5 µg.	10 µg.	20 µg.	40 µg.
0/3	12	12	13·5	9	8·5
1/2	9	11	14	5	3
2/4	20	25	25	21·5	—
3/4	12	19	15	8	—

In Table 5, which gives results obtained with auricles of which only a proportion had stopped, three points are evident. First, that the addition of small amounts of acetylcholine caused an increase in synthesis. Secondly, that the percentage increase was less than that produced by the same additions of acetylcholine in Table 4. Thirdly, that the addition of larger doses of acetylcholine caused a diminution in synthesis below the original value.

Each result in Table 4 and 5 was calculated as a percentage change from the original figure (taken as 100). The mean figure for each addition of acetylcholine was then plotted as shown in Fig. 6, curve *C*. Additions of acetylcholine up to 20 μg./g. produced effects in opposite directions on the synthesis by fresh auricles and on that by stopped auricles. While the synthesis in fresh auricles was depressed, that in stopped auricles was increased; with the larger addition of 40 μg./g., however, the curve for stopped auricles turned round, and synthesis was less than with half this dose. The curve for those auricles of which only a proportion had stopped was found to lie intermediate (curve *B*). With additions of acetylcholine up to 10 μg./g., the synthesis of these auricles increased; with further additions this synthesis was diminished to a similar degree as was that of the fresh auricles.

Some observations (which are being extended) have been made on the synthesizing power of auricles which were still beating at the end of 24 hr. These suggest that there is no gradual fall of synthesizing power, but that it remains within the normal limits of fresh auricles until the beat actually stops, after which it falls rapidly. The effect of acetylcholine on the synthesis by those powders from auricles, of which only a proportion had stopped, must therefore have been the algebraic sum of the increase of synthesis in the stopped auricles and the decrease of synthesis in the auricles still beating.

Effect of potassium. Since acetylcholine has the property of starting a stopped auricle, then, because of the similarity of its pharmacological action, KCl might also start it. Three experiments have been performed to test this. Amounts from 1 to 25 mg. KCl were added to a bath of 75 c.c., and failed to start the beat of the auricle. In one experiment the subsequent addition of 10 μg. acetylcholine started the auricle, and in a second, the subsequent addition of 100 μg. ACh started the auricle, in the third the subsequent addition of acetylcholine did not start the auricle, though it had started it before KCl was tried.

Experiments were also carried out to determine the effect of adding KCl to material prepared for incubation. Acetone powder from fresh auricles was used in two of the experiments, and the auricles freshly ground (after beating for 30 hr.) were used in the third. The results are shown in Table 6, in which amounts from 6 to 48 mg. KCl were added before incubation. The amount of 6 mg. is the normal addition of KCl. In the first experiment there was no inhibition of synthesis by KCl such as was always produced by acetylcholine, and indeed there was a slight increase. Because the amount of potassium

present in the activator (boiled brain extract) was not known, a second experiment was performed omitting the activator. In this experiment the biggest increase (33 %) was observed, but again there was no depression. The third experiment was carried out on auricles which were freshly ground with sand and activator, centrifuged, and aliquot amounts of the supernatant incubated. The figures for synthesis are therefore smaller, being calculated per g. fresh weight. A slight depression of synthesis was seen in this experiment in the sample to which 24 mg. KCl was added. We do not regard this single observation, which differs by not more than 12 %, as significant. We conclude that the action of KCl does not resemble the action of acetylcholine on synthesizing power.

TABLE 6. Effect of potassium chloride on acetylcholine synthesis (μg./g./75 min.)

	Amount of KCl added per 50 mg. powder or 0·35 g. fresh weight				
Preparation	0	6 mg.	12 mg.	24 mg.	48 mg.
Acetone powder + activator	17·5	22·5	25	26	—
Acetone powder + saline	15	15	17	20	16
Freshly ground + activator	—	6	6·15	5·25	—

Mann, Tennenbaum & Quastel (1939*a*) observed that KCl (0·027 M) increased the synthesis of acetylcholine when intact brain slices were used. Higher concentrations of KCl inhibited the synthesis. In brain brei, however, these authors (1939*b*) found KCl to have much less effect.

Action of adrenaline. The auricle which has stopped at the end of 24–30 hr. can often be started by the addition of adrenaline to the bath, and stops again soon after the adrenaline is removed. In some experiments adrenaline failed to start the auricle when subsequently acetylcholine was successful in doing so. In other experiments adrenaline started the auricle, and after washing out the bath, acetylcholine failed to start it. Torda & Wolff (1944) found that adrenaline increased acetylcholine synthesis in frog brain using the early method of Quastel, Tennenbaum & Wheatley (1936). We found that the synthesis of acetylcholine by a powder prepared from fresh auricle was not modified by the addition of adrenaline in amounts from 0·1 to 10 μg./50 mg. powder.

DISCUSSION

The observation has been made that when the auricles of the rabbit heart have ceased to beat after isolation in Tyrode solution for a period of 24–30 hr., the addition of acetylcholine starts the beat again, and further additions may increase it in rate and amplitude. Thus two effects of acetylcholine can be observed in the same auricle: (1) the usual inhibition which is observed throughout the period in which the auricle is beating, and (2) a stimulation of the stopped auricle shown by the resumption of contractions which gradually increase and may be augmented by a further addition of acetylcholine.

We have observed two other effects of acetylcholine, in parallel to these, in the course of investigating enzyme activity. In studying the power of synthesizing acetylcholine which the rabbit auricle possesses, we have found it to be high in the freshly beating auricle, and to be depressed by acetylcholine. On the other hand, we have found the synthesizing power of the stopped auricle to be low, and to be augmented by acetylcholine. In these observations we therefore have found a parallelism between the pharmacological effect of acetylcholine recorded on the drum and its biochemical effect exerted in the test-tube. Our experiments suggest that the enzymic activity in question, the synthesis of acetylcholine, has a close relation to the spontaneous activity of cardiac muscle. First, the synthesizing power is high in the freshly excised auricle which beats well, and in addition the action of acetylcholine which depresses this beat also depresses the synthesis in the fresh auricle. Secondly, the synthesizing power is low in the auricle which has ceased to contract, and the action of acetylcholine which starts the beat again augments the synthesis also. Thirdly, when the contractions have been restarted by acetylcholine, the synthesizing power approaches the value for fresh auricle.

The conception that acetylcholine may have another function in the body in addition to that of a transmitter of a nervous impulse has arisen for several reasons. Acetylcholine is present in the placenta (Chang & Gaddum, 1933) and synthesized there (Feldberg, 1945) though no transmission of nerve impulses is known to occur.

Evidence that acetylcholine plays a part in cardiac metabolism is suggested also by the experiments of Gremels (1936), who found that the continuous infusion of very small amounts into the heart-lung preparation caused a diminution in the oxygen consumption without any decline in the work done and therefore increased the efficiency.

A further indication is given by the pharmacological action of various substances on muscular tissue. Quinine and quinidine have long been known to prolong the refractory period of cardiac muscle, and in 1946 Dawes, using the isolated rabbit's auricle, found that this property is shared by procaine and many other substances having local anaesthetic and spasmolytic action. He drew attention to the fact that quinine and procaine antagonize the effect of acetylcholine in all forms of muscle. These observations suggested that the power of prolonging the refractory period might also be due to an antagonism to acetylcholine. In 1920, Drury, Lewis & Bulger showed that vagal stimulation reduced the refractory period, and Dawes observed that acetylcholine had the same effect.

The conception that acetylcholine plays a part in some process in cardiac muscle on which the excitability depends, and that this process is antagonized by substances like quinine, required the demonstration that acetylcholine possessed not only an inhibitory action on the heart, but also a stimulant

action. That such an action exists has been shown by Spadolini & Domini (1940), who perfused guinea-pig hearts with Ringer-Locke solution and found that low concentrations of acetylcholine stimulated the heart. The effect was abolished by atropine. McDowall (1946) has made similar observations in cats, rabbits and rats. Burn & Vane (1949) observed that auricles exposed to proguanil (paludrine) could be stimulated by acetylcholine, and when they had stopped, could actually be started by acetylcholine. What action proguanil may have in heart muscle is uncertain, and thus we were led to examine the effect of acetylcholine in auricles which had ceased to beat after many hours in Tyrode solution when no drug had been applied. Having found that acetylcholine started the beat, we proceeded to investigate its effect on acetylcholine synthesis by auricular tissue.

The view that acetylcholine plays a part in the contraction of cardiac muscle has been put forward by Koshtojanz (1938), on the ground that sodium fluoride applied to the frog heart reverses the effect of vagal stimulation. The view has also been put forward by Abdon (1945) who found, together with Hammarskjöld (1944), that rabbit hearts contain a precursor of acetylcholine which they extracted in a pharmacologically inactive form; on heating in acid solution, acetylcholine was liberated. In a later paper (Abdon & Borglin, 1945) on the effect of vagal stimulation no change in precursor content was found during the period in which the heart was arrested, but at the end of stimulation, when the heart beat was presumably good, the precursor content fell. Thus they were unable to demonstrate any diminution in the amount of precursor until vagal stimulation has been applied for at least 10 min. and they did not find any corrleation between this diminution and the heart's action. Later Abdon (1945) described experiments showing that in the isolated perfused rabbit heart the precursor content changed in parallel with the amplitude of the beat. The author does not explain how the amplitude of beat, plotted in his charts as percentage, was determined. However, assuming that a relation is established between precursor content and activity, it would remain to show that it was the precursor store which governed the activity, for the converse might equally be true. The conclusion that the precursor is continually broken down and resynthesized is a deduction since the experimental evidence is only concerned with precursor.

Our experiments have not been concerned with precursor store, but with synthesis of acetylcholine. Beznák (1934) was the first to observe that synthesis of acetylcholine occurred in cardiac tissue; he used the press juice of frog hearts which he incubated in the presence of eserine. He discussed the possibility that acetylcholine is present in the heart as a labile inactive precursor which gives off acetylcholine on vagal stimulation. Our experiments suggest that the normal rhythmic contractions of the heart depend on a synthesis of acetylcholine, which proceeds at a rate which is depressed by an addition of

acetylcholine from without (as when the vagus is stimulated). This might fit with the hypothesis of Brown & Eccles (1934) on the action of acetylcholine on the rhythmic mechanism of the pacemaker. They say 'The rhythmic mechanism of the pacemaker sets up a beat when its excitement reaches a certain threshold intensity. A.C. substance inhibits by acting as a quantitative antagonist to this excitement, the setting up of a beat being delayed until the excitement is built up to such an intensity that the uninhibited excitement attains a threshold value.' If in the foregoing quotation 'the synthesis of acetylcholine' is substituted for 'the building up of excitement', an approximation to our own conception is reached.

In considering the purely biochemical aspects of this work, it is not surprising to find that acetylcholine depresses its own synthesis in freshly excised tissue. It is common to find that addition of an end-product slows a chemical reaction. If, however, we turn to the increase of synthesis which acetylcholine brings about in the stopped auricle, we are faced by a phenomenon which so far as we know has no parallel, and for which we can offer no explanation.

The facts suggest, however, that in these biochemical observations we have a clue to the relation between motor and inhibitor effects. In this auricle these are not due to two distinct mechanisms, but are produced by one mechanism. Thus acetylcholine stimulates contraction when it is applied to auricles in which acetylcholine synthesis is proceeding at a low rate; acetylcholine inhibits contractions in auricles in which the synthesis is proceeding at a higher rate.

When the auricle is left to beat for 24-36 hr. in Tyrode solution, the beat eventually fails. We suppose that in this period some substances are diffusing out of the auricle into the bath, or else some store of material which supplies energy is gradually becoming exhausted. However, until the failure occurs there is no loss of synthesizing power, and any addition of acetylcholine still produces depression. When the beat stops and the synthesizing power rapidly declines, the addition of acetylcholine restores the beat and augments the synthesizing power. The effect of this addition is remarkable because it appears to start a process which then maintains itself, continuing after removal of the added acetylcholine. This strongly supports the view that the activity of the heart and the synthesis are inseparably linked, and that the activity is perhaps responsible for maintaining the synthesizing power.

SUMMARY

1. The auricles of the rabbit heart when freshly excised and placed in a bath of Tyrode solution will beat for periods of 24–36 hr. During this time the beat is depressed by the addition of acetylcholine. When the beat stops, it can be restarted by acetylcholine, and then continues for a long period. A further addition of acetylcholine may increase rate and amplitude.

2. The acetone-dried powder of freshly excised rabbit auricles incubated

according to the method of Feldberg & Mann (1946) synthesizes acetylcholine in varying amounts, the mean figure being 40μg./g. powder/75 min.

3. The powder from auricles, which have stopped beating after being kept in a bath of Tyrode for 24 hr., synthesizes less acetylcholine, the mean figure being 15μg./g. powder/75 min.

4. The addition of acetylcholine at the beginning of incubation inhibits the synthesis of acetylcholine by a powder made from fresh auricles. On the other hand, the addition of acetylcholine stimulates the synthesis of acetylcholine by a powder made from stopped auricles.

5. The view is put forward that the activity of the auricular muscle and the synthesis of acetylcholine are inseparably linked.

REFERENCES

Abdon, N.-O. (1945). *Acta Pharmacol. Toxicol.* **1**, 169.

Abdon, N.-O. & Borglin, N. E. (1945). *Acta Pharmacol. Toxicol.* **1**, 162.

Abdon, N.-O. & Hammarskjöld, S. O. (1944). *Acta physiol. Scand.* **8**, 75.

Beznák, A. B. L. (1934). *J. Physiol.* **82**, 129.

Brown, G. L. & Eccles, J. C. (1934). *J. Physiol.* **82**, 242.

Burn, J. H. & Vane, J. R. (1949). *J. Physiol.* **108**, 104.

Chang, H. C. & Gaddum, J. H. (1933). *J. Physiol.* **79**, 255.

Comline, R. S. (1946). *J. Physiol.* **105**, 6*P*.

Dawes, G. S. (1946). *Brit. J. Pharmacol.* **1**, 90.

Drury, A. N., Lewis, T. & Bulger, H. A. (1920). *J. Physiol.* **54**, 97*P*.

Feldberg, W. (1945). *J. Physiol.* **103**, 397.

Feldberg, W. & Mann, T. (1946). *J. Physiol.* **104**, 411.

Gremels, H. (1936). *Arch. exp. Path. Pharmak.* **182**, 1.

Koshtojanz, C. S. (1938). *C.R. Acad. Sci. U.R.S.S.* **19**, 315.

McDowall, R. J. S. (1946). *J. Physiol.* **104**, 392.

Mann, P. J. G., Tennenbaum, M. & Quastel, J. H. (1939*a*). *Biochem. J.* **33**, 822.

Mann, P. J. G., Tennenbaum, M. & Quastel, J. H. (1939*b*). *Biochem. J.* **33**, 1506.

Nachmansohn, D. & Machado, A. L. (1943). *J. Neurophysiol.* **6**, 397.

Quastel, J. H., Tennenbaum, M. & Wheatley, A. H. M. (1936). *Biochem. J.* **30**, 1668.

Spadolini, I. & Domini, G. (1940). *Arch. Fisiol.* **40**, 147.

Torda, C. & Wolff, H. G. (1944). *Proc. Soc. Exp. Biol., N.Y.*, **56**, 86.

J. Physiol. (1949) 108, 525–529 612.451.616.006.3

NORADRENALINE IN TUMOURS OF THE ADRENAL MEDULLA

By PAMELA HOLTON

From the Department of Pharmacology, University of Oxford

(*Received* 28 *December* 1948)

Blaschko (1942, 1948) has suggested that the immediate precursor of adrenaline in the body may be the primary amine, noradrenaline, which would be converted into adrenaline by *N*-methylation. One of the possible sites for this reaction is the suprarenal medulla, but although noradrenaline has been found in various parts of the body it has not yet been identified in normal suprarenal glands. A portion of human suprarenal tumour was sent to this laboratory and it was thought of interest to examine it for adrenaline and noradrenaline. It was found to contain a large amount of both amines. Two similar tumours which were examined later contained much noradrenaline and relatively little adrenaline.

Dr P. Glees who made a detailed examination of the first tumour stated that it consisted of chromaffin tissue and that he could find no nervous tissue. The other two tumours were examined by the hospital pathologists. All three tumours were typical phaeochromocytomata. The patients had suffered from attacks of high blood pressure.

MATERIAL AND METHODS

Collection of the material. The first tumour was sent by Dr E. G. Hall. Immediately after removal from the patient the tissue was placed in a jar and covered with N/10 HCl. The jar was brought to the laboratory in a freezing mixture of ice and salt (−15° C.) in a thermos flask.

The second tumour was sent by Dr G. M. Wilson of St Mary's Hospital, Paddington. It was also placed in N/10 HCl and frozen.

The third tumour was collected from Dr A. M. Joekes of the British Post-graduate Medical School. It was frozen but HCl was not added until it reached Oxford, 8 hr. after the operation

Preparation of the extracts. A weighed portion of each tumour was ground with sand and 10 ml. N/10 HCl/g. tumour. The acid extracts were heated in a boiling water-bath for 10 min. and then filtered. The filtrates (1 ml. = 0·1 g. tumour) were examined as follows.

RESULTS

Assay of adrenaline. West (1947) has shown that the rat's uterus and the frog's heart are much more sensitive to adrenaline than to noradrenaline so that these preparations can be used to determine the adrenaline in a mixture of the

two amines. In using the rat's uterus, a fixed amount of acetylcholine is added to the bath and allowed to act for a given time after which it is washed out. If adrenaline is added to the bath at a given interval before the dose of acetylcholine, the contraction produced by the latter is reduced to an extent which depends on the amount of adrenaline added. The three extracts were compared with adrenaline by this method. Fig. 1 is a record of the assay of extract 2. Extract 1 was also tested on the frog's perfused heart as shown in Fig. 2. The results of these assays are recorded in Table 1.

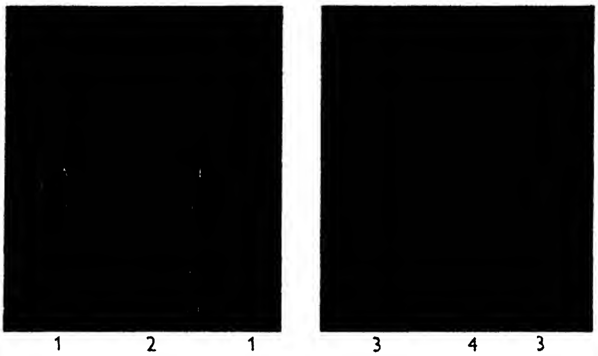


Fig. 1. Contractions of a rat's uterus in response to 5 μg. acetylcholine every 90 sec. Inhibition of contractions due to: (1) 0·001 μg. L-adrenaline; (2) 0·4 ml. 10^{-4} extract 2; (3) 0·3 ml. 10^{-4} extract 2; (4) 0·0012 μg. L-adrenaline.

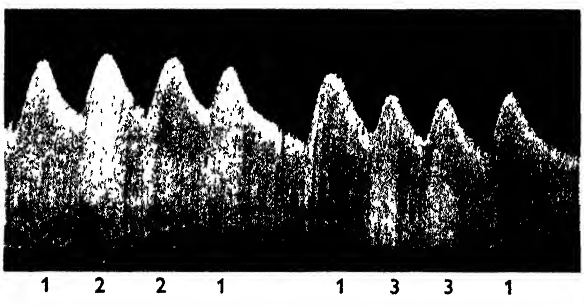


Fig. 2. Contractions of a frog's heart perfused with diluted Tyrode solution. Augmentation of contractions due to: (1) 0·025 μg. L-adrenaline; (2) 0·1 ml. 10^{-3} extract 1; (3) 0·05 ml. 10^{-3} extract 1.

Assay of adrenaline and noradrenaline together. Extracts 2 and 3 were assayed against L-adrenaline and DL-noradrenaline on the rabbit's duodenum. The comparison of extract 3 with DL-noradrenaline is shown in Fig. 3. It was found that both these extracts had more than ten times as much activity on the duodenum as on the rat uterus and that the duodenum was equally sensitive to

TABLE 1. Adrenaline assays

Extract	Preparation	Figure	Activity of the extracts	Adrenaline activity mg. L-adrenaline/g. of tumour: Limits	Adrenaline activity mg. L-adrenaline/g. of tumour: Estimate by interpolation
1	Rat's uterus	—	0·2 ml. 2×10^{-4} extract 1 >0·01 μg. <0·02 μg.	2·5–5·0	4
	Frog's heart	2	0·1 ml. 10^{-3} extract 1 >0·025 μg. 0·05 ml. 10^{-3} extract 1 <0·025 μg.	2·5–5·0	
2	Rat's uterus	1	0·4 ml. 10^{-4} extract 2 >0·001 μg. 0·3 ml. 10^{-4} extract 2 <0·0012 μg.	0·25–0·4	0·3
3	Rat's uterus	—	0·1 ml. 10^{-3} extract 3 <0·005 μg. >0·0025 μg.	0·25–0·5	0·4

L-adrenaline and DL-noradrenaline. Extract 1 was assayed against adrenaline on the duodenum and on the spinal cat's blood pressure; the blood-pressure assay is shown in Fig. 4. The results of all the assays of total activity (adrenaline plus noradrenaline) are in Table 2.

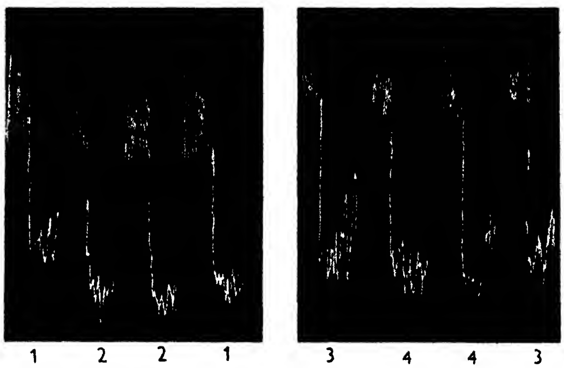


Fig. 3. Rabbit's isolated duodenum in Tyrode solution. Inhibition of spontaneous activity due to: (1) 0·3 ml. 2×10^{-2} extract 3; (2) 8 μg. DL-noradrenaline; (3) 7 μg. DL-noradrenaline; (4) 0·4 ml. 2×10^{-2} extract 3.

TABLE 2. Total activity assays (Adrenaline plus noradrenaline)

Extract	Preparation	Figure	Activity of the extracts	Total activity/g. of tumour: Limits	Total activity/g. of tumour: Estimate by interpolation
1	Rabbit's duodenum	—	0·2 ml. 5×10^{-3} extract 1 < 1·25 μg. adr. > 0·75 μg. adr.	7·5–12·5 mg. adr.	10 mg. adr.
	Spinal cat	4	1 ml. 2×10^{-2} extract 1 > 16 μg. adr. 0·7 ml. 2×10^{-2} extract 1 < 20 μg. adr.	8–14 mg. adr.	11 mg. adr.
2	Rabbit's duodenum	—	1 ml. 10^{-3} extract 2 > 0·7 μg. DL-noradr. 0·7 ml. 10^{-3} extract 2 < 1·0 μg. DL-noradr.	7–14 mg. DL-noradr.	10 mg. DL-noradr.
3	Rabbit's duodenum	3	0·3 ml. 2×10^{-2} extract 3 < 8 μg. DL-noradr. 0·4 ml. 2×10^{-2} extract 3 > 7 μg. DL-noradr.	8·75–13·3 mg. DL-noradr.	11 mg. DL-noradr.

Calculation of the noradrenaline content of the tumours. Since L-adrenaline and DL-noradrenaline have approximately the same activity on the rabbit's duodenum, the amount of noradrenaline in each extract is the total activity expressed as DL-noradrenaline less the activity due to adrenaline as shown by the rat's uterus assays. Table 3 shows the adrenaline content and the noradrenaline activity expressed as DL-noradrenaline of each tumour.

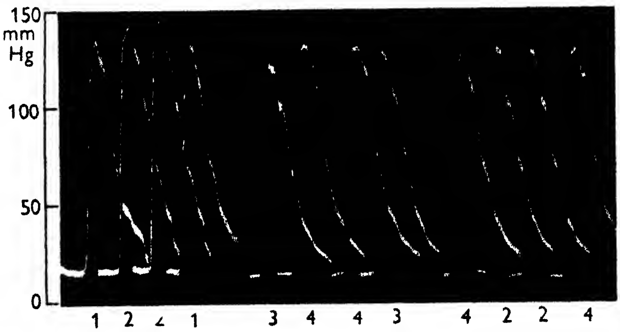


Fig. 4. Spinal cat's blood pressure: (1) 0·7 ml. 2×10^{-2} extract 1; (2) 20 μg. L-adrenaline; (3 16 μg. L-adrenaline; (4) 1 ml. 2×10^{-2} extract 1.

TABLE 3

(mg./g. of tumour.)

Tumour	Total	L-Adrenaline	DL-Noradrenaline
1	10	4	6
2	10·5	0·3	10
3	11	0·4	11

Identification of adrenaline and noradrenaline in extract 1. James (1948) has described tests for identifying pressor amines by paper chromatography and by using the enzyme polyphenolase prepared from *Atropa belladonna*. When he incubated extract 1 with polyphenolase there appeared a brown colour typical of noradrenaline, which soon faded and gave place to a pink colour typical of adrenaline. This reaction was also given by a mixture of the two synthetic amines and is specific for them. James also separated adrenaline and noradrenaline by paper chromatography. Phenol was used as a solvent and after 20 hr. the positions of the two amines were marked as pink spots when the paper was sprayed with potassium ferricyanide solution. Extract 1 gave two spots in positions typical of adrenaline and noradrenaline. These tests show that no other pressor amine was present in a significant amount.

DISCUSSION

The results described above show that the three tumours contained large amounts of noradrenaline. The first tumour also contained adrenaline in a quantity of the same order as, but less than that of, noradrenaline. DL-Noradrenaline has slightly greater pressor activity than L-adrenaline (West, 1947) and it seems likely that the blood pressure crises caused by the three tumours were mainly due to the release of noradrenaline. There are indications, on the other hand, that in some cases adrenaline may be responsible for the high blood pressure, since Goldenberg, Snyder & Aranow (1947) found that the effect of the adrenolytic drug 993 F in patients with phaeochromocytomata resembled antagonism of adrenaline rather than of noradrenaline.

SUMMARY

Large amounts of noradrenaline have been found in three tumours of the adrenal medulla.

Note added in proof. Since this paper was submitted a publication of von Euler & Hamberg (1949) has appeared in which the identification of noradrenaline in the suprarenals of cattle is reported. My attention has also been drawn to a recent communication by Schümann (1948) in which evidence is produced of the presence of noradrenaline in pigs' suprarenals.

I am grateful to Dr E. G. Hall, Dr G. M. Wilson and Dr A. M. Joekes for supplying portions of the tumours and to Dr P. Glees and Dr W. O. James for examining one of the tumours.

My thanks are also due to Prof. J. H. Burn who suggested this work.

REFERENCES

Blaschko, H. (1942). *J. Physiol.* **101**, 357.

Blaschko, H. (1948). *The Hormones: Physiology, Chemistry and Applications*, **2**, ch. 25*b*. New York: Academic Press Inc. (In the Press.)

von Euler, U. S. & Hamberg, U. (1949). *Nature, Lond.*, **163**, 642.

Goldenberg, M., Snyder, C. H. & Aranow, H. Jr. (1947). *J. Amer. pharm. Ass.* **135**, 971.

James, W. O. (1948). *Nature, Lond.*, **161**, 851.

Schümann, H. J. (1948). *Klin. Wschr.* **26**, 604.

West, G. B. (1947). *J. Physiol.* **106**, 418.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

26 *June* 1948

A servo-stimulator. By W. A. H. Rushton. *Physiological Laboratory, University of Cambridge*

It is often convenient to be able to deliver *current* pulses of determined intensity and wave form, unaffected by the resistance and reactance of the tissue. The present device draws no current at input and will deliver at output about 10 mA. (or much more with suitable output valve and power source). It has proved satisfactory in a variety of applications.

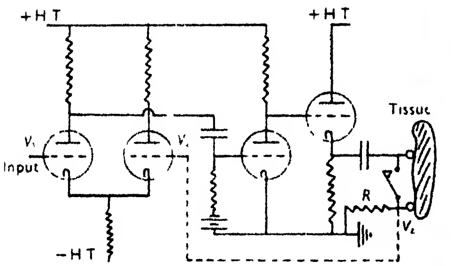


Fig. 1.

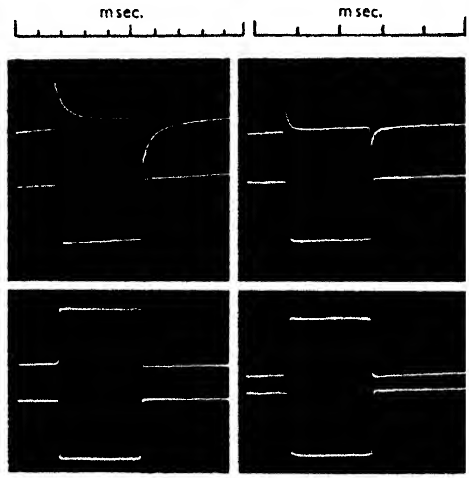


Fig. 2.

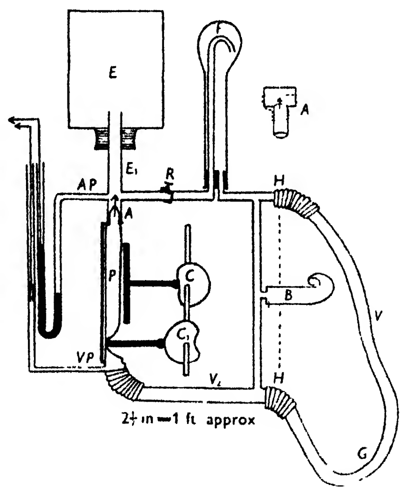
Principle. The required wave form is applied as a voltage pulse V_1 to one grid of a differential input $V_1 V_2$ with common cathode resistance. Supposing V_1 rises more than V_2, a greatly amplified potential rise is applied to the last valve (a cathode follower with independent high tension) and a positive pulse flows through condenser, tissue and R to earth. If R is not too small the potential across it will be large, and this, applied to the input V_2, will instantly neutralize the differential output. It follows that V_2 must always remain at the same potential as V_1, since any difference will be annihilated by the pulse generated. But V_2 is proportional to the current through R which is the current through the tissue; therefore this is proportional to V_1 independent of impedance.

Performance. A current pulse was led through a human arm between moist pads and very polarizable electrodes. Fig. 2 shows four records; those on the left are with large pads, the others with small. In each frame the lower trace shows the applied wave V_1, a square pulse. The upper trace shows the potential drop across 1000 ohms in series with the arm, and is hence proportional to the current through the arm. In the upper frames a constant *voltage* pulse proportional to V_1 was applied to the arm and series resistance. The current shows the familiar polarization picture. In the lower frames the servo-stimulator was used, and the current form is a good image of the voltage input.

Demonstration to the Society. To show that the performance does not just depend upon a square wave input, V_1 is led from an amplified action potential of a frog's nerve. The simultaneous records of the frog's action potential and the resulting current through the human arm are seen to be exact reflexions.

An artificial circulation. By R. J. S. McDowall. *King's College, London*

This artificial circulation is a robust piece of apparatus which has been in use by medical students for a year and is one by which the main mechanical features of the circulation of the blood may be shown.



The pump has the special feature that its output can be shown to depend both on its input and to a limited extent on the frequency of its stroke. It consists essentially of a piece of flattened bicycle 1 in. inner tubing 4 in. long which is compressed by a moving plate controlled by a cam (C). A large inlet 'valve' of little resistance is provided by the closure of the inlet by a narrow plate driven by another cam (C_1) on the same shaft just before the main stroke of the pump. A very robust and simple exit valve at A is provided by a piece

of inner tubing stretched over a piece of brass tubing into the outer end of which is inserted a wider flat piece of bakelite. A visible flow indicator (*F*) in the system is provided by a bent tube inside a small Kjeldahl flask, the flow being directed against the side of the flask to avoid frothing.

The rate of the pump can be altered by changing the starting resistance of the motor driving the cam-shaft.

The 'arterial' pressure (*AP*) taken on a mercury manometer can be altered up to 200 mm. Hg by changing the filling of the heart, its rate within limits and by varying the resistance of the system (a clip at point *R*). Increased filling of the heart is produced by raising the hinged part beyond *H...H* of the system to the horizontal or by compressing the 'blood depot' (*B*) which is a piece of inner tube which can be rolled up. The air pressure can be adjusted in the bottle *E* so that the changes in 'heart' output become visible.

The 'venous pressure' (*VP*) taken on a water manometer (a narrow burette with a fountain pen cap as a piston) can be shown to be affected by the amount of circulating fluid, by the capacity of the system, which can be increased by unrolling *B*, by the peripheral resistance and by the output of the pump. If the pump is slowed or stopped, the 'venous pressure' rises markedly as in cardiac disease.

A reduction of the elasticity of the system provided by the inverted bottle *E* is effected by pinching tube E_1 and is shown to increase the pulse pressure and render the flow in the flow-meter (*F*) intermittent.

A lever system (not shown in diagram) from the rubber valve chamber at *A* may be added to record the rapid changes in the 'arterial pressure' better than the mercury manometer.

For making original forms of the pump and the exit valve I am particularly indebted to Dr A. E. Schuster.

The effect of D.F.P. upon the electrocardiogram of the isolated rabbit heart.

By J. A. C. Knox, J. P. Quilliam and F. G. Strong. *King's College, London*

Quilliam & Strong (1947) have shown that the sensitivity of the isolated rabbit heart to acetylcholine can be increased between 10- and 100-fold following the injection of 0·25 mg. of D.F.P. (di-isopropyl fluorophosphonate) into the perfusate.

It was found that the injection of D.F.P. alone was accompanied by a transient diminution in amplitude of the mechanical record of the heart beats from which recovery was complete in about 2 min. It was therefore of interest to learn whether the injection of D.F.P. was accompanied by any changes in the electrocardiogram and a series of such e.c.g.'s will be demonstrated.

Leads were taken by non-polarizable electrodes from the right auricular appendix and the posterior surface of the left ventricle. The action potentials were amplified by a resistance-capacity coupled circuit including a Toennies balanced input and were observed on one cathode-ray tube and photographed from a repeater tube. In all cases the mechanical record showed that the dose of D.F.P. employed had sensitized the heart to the action of acetylcholine.

(1) *Electrocardiographic changes following the injection of* 0·025 *ml. of propylene glycol, the solvent used for D.F.P.* The propylene glycol caused only minor changes in the e.c.g. These included a slight prolongation of the *P-R* interval, a transient broadening and splintering of the *P* or *QRS* complexes of small extent and slight changes in the amplitude of *T*.

(2) *Electrocardiographic changes following the injection of* 0·25 *mg. of D.F.P.* (*i.e.* 0·025 *ml. of a* 1 % *solution in propylene glycol*). When D.F.P. was injected into a fresh perfused heart, the electrocardiographic changes were slight and were similar in character to those described in the previous section. They could, therefore, be attibuted entirely to the propylene glycol in which the D.F.P. was dissolved. Occasionally, when D.F.P. was injected towards the end of a prolonged experiment, more definite effects were produced including nodal rhythm and auriculo-ventricular dissociation. In every case, however, in which these marked effects appeared, the heart was in poor condition and had been previously subjected to repeated doses of acetylcholine. Therefore they cannot be regarded as typical D.F.P. effects.

(3) *Electrocardiographic changes following the injection of acetylcholine before and after D.F.P.* With increasing dosage the effects of acetylcholine on the e.c.g. varied from simple prolongation of the *P-R* interval, through partial or complete heart block, to ventricular standstill of varying duration. More rarely, auricular standstill also occurred. In a small series, Noth, Essex & Barnes (1939) found similar changes following intravenous injection of acetylcholine in the intact dog. In our experiments, as might be expected, D.F.P. potentiated the electrocardiographic effects. In general, a dose of acetylcholine given after D.F.P. had approximately the same effects as one at least ten times as great given before the D.F.P.

In conclusion it would appear that D.F.P. injected alone, does not cause any significant changes in the e.c.g. With intramuscular injections of 1–3 mg. of D.F.P. in man, Comroe, Todd & Koelle (1946) found no alteration in the e.c.g. In our experiments it can be assumed that a much higher concentration of D.F.P. would reach the cardiac muscle and even under these circumstances no significant electrocardiographic changes appeared.

REFERENCES

Comroe, J. H., Todd, J. & Koelle, G. R. (1946). *J. Pharmacol.* **87**, 281.

Noth, P. H., Essex, H. E. & Barnes, A. R. (1939). *Proc. Staff Meet. Mayo Clin.* **14**, 348.

Quilliam, J. P. & Strong, F. G. (1947). *J. Physiol.* **106**, 23 *P*.

A method of measuring biological outflows using a simple siphon and electronic recording. By J. P. Quilliam. *King's College, London*

The simple siphon type of flow measurer used by Knowlton & Starling (1912) in the heart-lung preparation with air transmission of each siphoning event was developed by Gunn (1913) and later by Symes (1918). In an attempt to overcome some of the difficulties in the construction and the use of such types of apparatus, advantage was taken of the introduction of the electronic drop recorder (e.g. Harris, 1931; Winton, 1936) to record each siphoning event. Both the siphons and the electronic recorder demonstrated can be readily constructed in any physiological laboratory.

The fluid flowing from a tissue is collected in a funnel leading to one limb of an ∽-shaped glass tube. When just sufficient fluid has been collected to overfill this and the adjacent limb, the fluid, commencing to overflow down the third limb, initiated a siphonating action emptying the whole ∽-tube. The siphoned fluid is directed to pass over two electrodes in an electronic circuit embodying an indirectly heated cathode valve (KT 33 C) which is normally overbiased until the siphoned fluid momentarily short-circuits the electrodes. The transient surge of current set up by the fluid short-circuit of the excess of bias is used to work a master relay which can operate as required an electromagnetic signal writing on a kymograph, an electromagnetic counter or a light signal so recording each siphoning event. The electronic circuit has been designed so that electrolysis of the fluid is minimal and the apparatus can be worked off the 100 V. d.c. supply mains.

By constructing siphons of different sizes, various outflows may be measured. With the smallest siphon in this series a flow that is just too rapid to be measured with a drop recorder may be measured satisfactorily. With more rapid outflows, a larger siphon is used. The bends of the siphon tube should be easy and free from constrictions and the third limb should be of a bore to permit the rapid and free discharge of the siphoned fluid.

REFERENCES

Gunn, J. A. (1913). *J. Physiol.* **47**, iii.

Harris, D. T. (1931). *J. Physiol.* **71**, 22*P*.

Knowlton, F. P. & Starling, E. H. (1912). *J. Physiol.* **44**, 206.

Symes, W. L. (1918). *J. Physiol.* **52**, lii.

Winton, F. R. (1936). *J. Physiol.* **87**, 20*P*.

Double action of acetylcholine on cardiac and vascular tissue.

By EDITH BÜLBRING and J. H. BURN. *Department of Pharmacology, University of Oxford*

Burn & Vane have recently found that acetylcholine has a double action on the isolated rabbit auricles (when treated with paludrine), on strips of intestine and of rat uterus.

We have now observed that the contractions of the isolated rabbit auricles gradually diminish during 24 hr. and then stop, no drug having been applied. When stopped they can be restarted by the addition of acetylcholine in small doses. When the beat is thus re-established, acetylcholine resumes its inhibitory action.

When the vessels of the rabbit ear are perfused with Locke solution, during the first day the action of acetylcholine is dilator. After 24 hr. the action of acetylcholine becomes constrictor; if acetylcholine is now continuously perfused through the vessels, the injection of a single dose once more produces dilatation. These phenomena, which are similar in heart and blood vessels, demonstrate the close relation between motor and inhibitor actions of acetylcholine.

Reduction in coronary flow by pituitary (posterior lobe) extract in relation to the action of nicotine.

By EDITH BÜLBRING and J. M. WALKER. *Department of Pharmacology, University of Oxford*

The smoking of one or two cigarettes inhibits water diuresis in man, and the inhibition can be matched by giving nicotine intravenously or pituitary (posterior lobe) extract. The inhibition is accompanied by an increased chloride excretion. Since Pickford (1947) has proved that acetylcholine produces this effect in dogs by liberating the antidiuretic hormone, it is probable that nicotine inhibits diuresis in the same way.

The antidiuretic and pressor hormones are believed to be identical, and the hormone liberated by smoking may cause coronary constriction. One or two cigarettes in man release 50–100 milliunits. In a blood volume of 5 l. this corresponds to 10–20 milliunits/l.

We have observed that in the dog heart-lung preparation, using 1 l. of blood, the threshold dose of pituitary extract causing a reduction of coronary flow is 10–20 milliunits.

REFERENCE

Pickford, M. (1947). *J. Physiol.* **106**, 264.

Relationship between force and speed in the human muscle. By
D. R. WILKIE (introduced by C. LOVATT EVANS). *Department of Physiology, University College, London*

It is well known that a muscle can shorten more quickly against a small force than against a large one. Hill (1938) has shown, both by heat-production studies and direct mechanical experiments, that in isolated muscles these two variables are related by the equation

$$(P+a)\,(V+b)=\text{constant},$$

where P = force, V = velocity, and a and b are constants.

The work described in this communication extends observation of the force-velocity relationship to the more complex circumstances of human voluntary movement.

It is shown that in maximal flexions of the elbow, the relationship between force and velocity (both measured at the hand in a direction parallel with the upper arm) is represented adequately by Hill's equation.

To interpret this result in terms of the properties of human *muscle* involves two difficulties:

(1) That the muscles are not arranged geometrically in any simple way.

(2) That their degree of activation may be varying during the movement.

These two difficulties are discussed.

REFERENCE

Hill, A. V. (1938). *Proc. Roy. Soc.* B, **126**, 136.

Chemoreceptors on the tongue of the toad. By
B. L. ANDREW (introduced G. H. BELL). *Physiology and Biochemistry Department, University College, Dundee*

Afferent nerve impulses from chemoreceptors of the lingual epithelium may be picked up from the lingual branch of the glossopharyngeal nerve. The nerve trunk gives off fine branches within the tongue and from these the electrical responses of a single active fibre may be recorded.

The chemoreceptors appear to be of a single type responding to salt solutions. Approximately isotonic salt solutions evoke no discharge, but hypertonic solutions above a threshold which varies from receptor to receptor produce an irregular discharge which has a slow rate of adaptation.

In addition, some receptors discharge when hypotonic salt solutions are applied.

All are relatively insensitive compared with mammalian lingual salt receptors.

Effect of increasing plasma levels on tubular excretion of diodone.
By J. A. Barclay, W. T. Cooke and G. de Muralt. *Department of Physiology and Medicine, University of Birmingham*

According to Smith (1938), with increase of plasma diodone, a level is reached at which the amounts secreted by the tubules become maximal and not affected by further increases in plasma level. Reinvestigation of this concept in subjects with normal and damaged kidneys indicates that values for Tm Diodone (as calculated by the usual formula) diminish with further increase of plasma levels and in some subjects may become negative.

REFERENCE

Smith, H. W. (1938). *J. clin. Invest.* **17**, 263.

Effects of electrical stimulation of the aortic nerve in animals under nembutal and chloralose anaesthesia.
By E. Neil, C. R. M. Redwood and A. Schweitzer. *Departments of Physiology, School of Medicine, Leeds, and University College, London*

Rectangular wave stimulation of the aortic nerves, with varying frequencies and pulse durations, caused a fall in the arterial blood pressure in nembutalized cats and rabbits. In nembutalized dogs the effects of stimulation depended upon the pulse duration of the stimulus.

In chloralosed cats stimulation of the right aortic nerve caused a rise in blood pressure, independent of the pulse duration employed. Stimulation of the left aortic nerve produced variable results.

In chloralosed rabbits a fall of blood pressure invariably resulted from aortic nerve stimulation.

In chloralosed dogs nerve stimulation produced variable results.

The depressor response to aortic nerve stimulation in nembutalized cats was converted to a rise of blood pressure by intravenous injection of $\geqslant 0{\cdot}05$ g. chloralose.

Injection of chloralose into nembutalized rabbits did not affect the depressor response to aortic nerve stimulation. In nembutalized dogs aortic nerve stimulation after chloralose injection more commonly caused pressor responses.

Further observations on the structure of frog nerve lipoprotein.

By J. J. Elkes and J. B. Finean. *Department of Pharmacology, University of Birmingham*

Low-angle X-ray diffraction patterns of perfused frog sciatic have been obtained, and the effects of slow drying and change of temperature studied. Normal spacings are observed at 33·6, 42·5, 55·6, 85·4 and 172 A. units. The first four of these may reflect orders of the fifth (Schmitt, Bear & Palmer, 1941).

During the first stage of drying the 85·4 A. spacing shrinks to 79 A. There is also an independent increase in intensity of the 55·6 A. spacing. These changes are reversible.

Carried beyond this point drying results in a further shrinkage of the 79 A. dimension to 74 A. There is also a breaking up of the 55·6 A. band into two spacings at 44·1 and 61 A. These show poor orientation, and are of the order encountered in dried ether extract of nerve. The changes now are irreversible.

The myelin unit may be built of lipid and lipid-protein sub-units, which vary in their susceptibility to hydration. The end result of drying may reflect a phase separation.

REFERENCE

Schmitt, F. O., Bear, R. S. & Palmer, K. J. (1941). *J. cell. comp. Physiol.* **18**, 31.

The dependence of neuromuscular transmission on glucose.

By I. Hajdu and R. J. S. McDowall. *King's College, London*

If a rat diaphragm preparation in Tyrode solution is deprived of glucose after a variable period sometimes lasting several hours, there is an augmentation of contraction followed by a depression and ultimate cessation of response to nerve stimulation (McDowall & Shafei, 1947). It is now shown that this depression is really due to a neuromuscular block, for the muscle remains excitable like a denervated muscle to stimuli of long duration, but not to those of short duration. That the block is not due simply to the repeated stimulation is shown by its reversibility by glucose. Lack of calcium has no effect on the contraction of the muscle after such a block which herein differs from a curare block. A block of synaptic transmission in ganglia due to lack of glucose has already been described by Kahlson & MacIntosh (1939).

Such a preparation blocked by glucose lack provides a very convenient method for the study of muscle apart from nerve.

REFERENCES

Kahlson, G. & MacIntosh, F. C. (1939). *J. Physiol.* **96**, 277.
McDowall, R. J. S. & Shafei, A. Z. (1947). *J. Physiol.* **106**, 2 *P*.

Some actions of calcium and potassium in the rat diaphragm.

By I. HAJDU and R. J. S. McDOWALL. *King's College, London*

The innervated rat diaphragm is extremely sensitive to calcium deprivation. If the amount of calcium is reduced to a quarter or less of that in Tyrode solution, there is at first an augmentation followed by a depression and neuromuscular block. The latter was discovered by Locke (1894) and confirmed by Mines (1911) in the frog. Reduction of the calcium to half greatly increases the sensitivity of the preparation to curare. Complete absence of calcium reduces the augmentation. The depression does not occur in the denervated preparation, but is present after neuromuscular block by curare.

Deprivation of potassium results in a very slight depression, but excess leads to augmentation followed by a complete failure of response to direct and indirect stimuli. This occurs after denervation and after curare and is antagonized by calcium and by *adrenaline*.

REFERENCES

Locke, F. S. (1894). *Zbl. Physiol.* **8**, 166.
Mines, G. R. (1911). *J. Physiol.* **42**, 251.

An 'atropine-like' effect exerted by eserine in the isolated rabbit heart which has received D.F.P.

By J. P. QUILLIAM and F. G. STRONG. *King's College, London*

The isolated perfused rabbit heart which has received a dose of D.F.P. such that more D.F.P. will cause no further increase in the sensitivity of the preparation to acetylcholine may be said to be 'fully fluorized'. Eserinization of the perfusion fluid of the fully fluorized heart leads to some reduction in its sensitivity to acetylcholine, a change reminiscent of the effect of small doses of atropine.

Full-fluorization has no action upon the sensitivity of the heart to pilocarpine or to arecoline, but, on eserinization of the fully fluorized heart, an 'atropine-like' action of eserine can also be shown in respect to pilocarpine and to arecoline. A return to the normal perfusion fluid for the preparation restores, in about 1 hr., the sensitivity of the heart to acetylcholine, pilocarpine and arecoline.

Prostigmine, also, may exert an 'atropine-like' effect upon the sensitivity of the fully fluorized heart.

The inhibitory effect of fluoroacetate and the tricarboxylic cycle.

By C. Liébecq and R. A. Peters. *Department of Biochemistry, University of Oxford*

Bartlett & Barron (1947) have advanced the hypothesis that fluoroacetate ($F.CH_2.COONa$) is a competitive inhibitor for acetate, and that it so prevents entry of acetate into the reactions of the tricarboxylic cycle. We now have evidence that it also prevents reactions within the cycle itself. In a centrifuged homogenate from kidney (guinea-pig) the oxidation of fumarate is inhibited with accumulation of citrate, whereas citrate is normally well oxidized. This participation in the cycle is consistent with the original evidence of Swarts (1896) that the —C—F bond is very stable, with the findings of Buchanan, Sakami, Gurin & Wilson (1945) that acetate enters the cycle, and with the evidence of Saunders (1947) and collaborators that the active poison is $F.CH_2.CO$, and that even-numbered ω-fluorocarboxylic esters are toxic and odd-numbered not.

REFERENCES

Bartlett, G. R. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 67.

Buchanan, J. M., Sakami, W., Gurin, S. & Wilson, D. W. (1945). *J. biol. Chem.* **159**, 695.

Saunders, B. C. (1947). *Nature, Lond.*, **160**, 179.

Swarts, F. (1896). *Bull. Acad. roy. Belg.* IIIe, **31**, 675.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

23–24 *July* 1948

An improved method of preparing fixed and stained whole mounts of tissue cultures grown in plasma coagula. By ANNABELLE COHEN and CHARITY WAYMOUTH. *Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London, S.W.* 3

Increasingly time-consuming and complicated methods have been proposed (e.g. Fischer, 1930; Parker, 1938; Tompkins, Cunningham & Kirk, 1947; Earle, 1944, 1947) for the preparation of fixed and stained mounts from whole tissue cultures, in attempts to overcome the difficulties caused by the density and impermeability of the fixed coagulum. Prolonged staining and the subsequent elaborate efforts to secure contrast between the staining of the cells and that of the coagulum have been dispensed with by introducing the step of drying the whole tissue culture after fixation. The coagulum dries down on to the cover-slip, giving a thin preparation which can be stained, dehydrated, cleared and mounted as rapidly as can a thin tissue section. The cells stain sharply and evenly without distortion or shrinking and background staining is markedly reduced.

REFERENCES

Earle, W. R. (1944). *J. Nat. Cancer Inst.* **4**, 539.
Earle, W. R. (1947). *J. Nat. Cancer Inst.* **8**, 83.
Fischer, A. (1930). *Gewebezüchtung.* Munich: Müller and Steinicke.
Parker, R. C. (1938). *Methods of Tissue Culture.* New York: P. B. Hoeber Inc.
Tompkins, E. R., Cunningham, B. & Kirk, P. L. (1947). *J. cell. comp. Physiol.* **30**, 1.

An electronic heart beat frequency meter. By W. R. BEAKLEY and J. D. FINDLAY (introduced by R. C. GARRY). *The Hannah Dairy Research Institute, Kirkhill, Ayr*

Bell & Knox (1938), Bell & Weir (1939) and Knox (1940) made an electronic device to record heart beat frequency in man during exercise. Our instrument has been designed for the same purpose in animals subjected to tropical conditions in a psychrometric room. The heart beat frequency is displayed in a room remote from the experimental animal.

Three leads, two dorsally and one on the thorax over the apex beat of the heart, are attached to the animal using soft soap as electrode paste (Bell, Knox & Small, 1939). The input from these leads, balanced with respect to

earth, is connected to a paraphase amplifier. The gain and bandwidth of the amplifier are controllable and provision is made to connect a filter tuned to 50 cycles for the suppression of any out-of-balance mains voltage picked up by the animal. A monitoring point is provided so that the cardiac waveform may be observed on an oscilloscope.

The e.m.f. corresponding to the *QRS* peak in the amplifier output has a much greater amplitude than that of any other part of the waveform and is used to trigger a multivibrator providing a voltage pulse of definite amplitude and duration and also a current pulse sufficiently large to operate a magnetic relay. This latter is used to provide marks corresponding to each heart beat on a kymograph drum.

The voltage pulse is used to control the anode current of a valve so that this current is directly proportional to the number of heart beats per minute. After suitable smoothing, the voltage obtained from this current after passing through a 2 megohm resistance, is taken to the grid of a valve in a cathode follower circuit so that the output can be measured on a low-resistance voltmeter.

The meter is calibrated from 40 to 140 heart beats per minute and a practically linear relationship between heart rate and meter deflexion is obtained over the whole scale with an accuracy of ± 1 heart beat per minute. The range of the meter can be extended.

REFERENCES

Bell, G. H. & Knox, J. A. C. (1938). *J. Physiol.* **93**, 36 *P*.

Bell, G. H., Knox, J. A. C. & Small, A. J. (1939). *Brit. Heart J.* **1**, 229.

Bell, G. H. & Weir, J. B. de V. (1939). *J. Physiol.* **96**, 31 *P*.

Knox, J. A. C. (1940). *Brit. Heart J.* **2**, 289.

Some features of the response of the heart rate to exercise in patients with auricular fibrillation. By J. A. C. Knox

In auricular fibrillation the maximum heart rates reached during a standard exercise were much higher than in normals or in patients with simple valvular disease. No correlation was found between any of the heart-rate indices (initial rate, maximum rate, acceleration, percentage increase, etc.) and the clinical condition of the various patients. In spite of the complete irregularity of the ventricular rate, the response to exercise in a given individual was reasonably constant.

In some cases a brief fall in heart rate occurred at the beginning of exercise. An almost constant feature was a sudden delayed acceleration of the heart rate commencing about 12 sec. after exercise had begun. Electrocardiograms taken during the exercise showed that the delayed acceleration was not due

to a temporary change from fibrillation to flutter. It would appear to be a characteristic of hearts in which the sino-auricular node is no longer acting as pacemaker.

Effect of surface-active substances on the liberation of enzymes from rabbit polymorphonuclear leucocytes. By R. J. Rossiter (introduced by G. E. Hall). *Department of Biochemistry, University of Western Ontario, London, Canada*

Cell-free preparations have been obtained by adding surface-active substances to suspensions of the cells (obtained by the method of de Haan, 1918). In the presence of saponin, a tributyrinase and an alkaline phosphomonoesterase were liberated from the cells. If the cells were suspended in isotonic saline, most of the tributyrinase activity remained in the cells after centrifuging, whereas if saponin had been added, most of the activity was in the supernatant. The same was true for the alkaline phosphomonoesterase, but, unlike the tributyrinase, the phosphomonoesterase activity was far from maximal in cell suspensions. However, when saponin was added to centrifuged cells that previously had been suspended in isotonic saline, full activity was observed.

Similar results were obtained using sodium taurocholate, sodium desoxycholate and very small quantities of the extremely active preparation 'alkyl sulphate'. It should be stressed that the liberation of enzymes from rabbit polymorphonuclear leucocytes is not an all-or-none phenomenon as is the lysis of red cells.

REFERENCE

de Haan, J. (1918). *Arch. néerl. Physiol.* **2**, 674.

Esterase of rabbit polymorphonuclear leucocytes. By R. J. Rossiter and Esther Wong (introduced by G. E. Hall). *Department of Biochemistry, University of Western Ontario, London, Canada*

Washed rabbit polymorphonuclear leucocytes were found to contain an active esterase, thus confirming the observations of Fleischmann (1928) and Barnes (1940). These workers, however, referred to the enzyme as a *lipase*. We have shown that the enzyme is capable of hydrolysing simple triglycerides and simple methyl esters (up to C_8 have been tested), but not higher glycerides such as triolein. The enzyme should, therefore, be referred to as an *esterase* rather than a *lipase*.

It can be seen that the rabbit polymorph hydrolysed neither acetylcholine, acetyl-β-methylcholine nor benzoylcholine and, therefore, contains neither

'true' cholinesterase, 'pseudo'-cholinesterase nor specific benzoylcholinesterase.

The kinetics of rabbit polymorphonuclear leucocyte esterase have been studied using both tributyrin and methyl butyrate as substrate. The enzyme was inhibited by eserine, tri-*o*-cresyl phosphate, arsanilic acid, acetophenone and fluoride at low concentrations and by urethane, quinine and mepacrine at higher concentrations.

REFERENCES

Barnes, J. M. (1940). *Brit. J. exp. Path.* **21**, 264.
Fleischmann, W. (1928). *Biochem. Z.* **200**, 25.

l-Adrenaline and *l*-noradrenaline. By J. D. P. Graham. *Department of Materia Medica, Glasgow*

According to West (1947) *dl*-noradrenaline is a stronger pressor agent than *l*-adrenaline.

The relative activity of *l*-adrenaline and *l*-noradrenaline was tested on the following preparations. The pressor response to *l*-noradrenaline in spinal cats was 165% of that of *l*-adrenaline which agrees with the assay of Tainter, Tullar & Luduena (1948). In the isolated ileum of rabbit *l*-noradrenaline was more than twice as potent as *l*-adrenaline as an inhibitor. *l*-Adrenaline (10^{-9}, 10^{-10} or more) had an inhibitor effect on isolated rectal caecum of hen, whereas *l*-noradrenaline in the same concentration had a motor effect. This preparation serves as a method of distinguishing the two, as does the isolated duodenum of duck which usually has a biphasic response to *l*-adrenaline (10^{-8}) and a purely motor response to *l*-noradrenaline.

REFERENCES

Tainter, M. L., Tullar, B. F. & Luduena, F. P. (1948). *Science*, **107**, 39.
West, G. B. (1947). *J. Physiol.* **106**, 418.

Vasopressor action of acetylcholine in the atropinized cat. By David Burns and the late John Secker. *Department of Physiology, Medical School, King's College, Newcastle-upon-Tyne*

Danielopolu (1946) found that acetylcholine given intravenously to fully atropinized dogs produced a rise of blood pressure which was not shown after adrenalectomy. Haney & Lindgren (1945), using unanaesthetized atropinized dogs with denervated hearts, got cardiac acceleration after acetylcholine. Bilateral removal of the adrenal medulla had no effect on this. In the isolated heart perfused with Tyrode solution, Hoffmann, Hoffmann, Middleton & Talesnik (1945) found that, after atropinization, acetylcholine produced a

cardiac acceleration which was wiped out by ergotamine. Our experiments show that acetylcholine after atropinization has a distinct pressor effect which is reduced by adrenalectomy and restored by administration of adrenocortical extracts. (Eucortone, *Allen & Hanbury*, or Eschatin, *Park Davies*.)

REFERENCES

Danielopolu, D. (1946). *C.R. Soc. Biol., Paris*, **140**, 298.

Haney, H. F. & Lindgren, A. J. (1945). *Amer. J. Physiol.* **145**, 177.

Hoffmann, F., Hoffmann, Elena J., Middleton, S. & Talesnik, J. (1945). *Amer. J. Physiol.* **144** 189.

Broncho-constriction in isolated perfused dog lungs in response to inhalation of ammonia. By JEAN BANISTER and CATHERINE O. HEBB. *Department of Physiology, Edinburgh University*

When isolated blood-perfused dog lungs (under negative pressure ventilation) are made to inhale NH_3 vapour, broncho-constriction occurs. Threshold doses vary for different preparations from 3 to 8 c.c. of *c.* 1 : 1 air-NH_3 mixture; 4–5 times a threshold dose gives maximal constriction. Eserine, added to the perfusate (concentration 1 : 200,000), potentiates the response so that threshold doses have a maximal action. This potentiation is nullified by atropine; and in atropinized, eserinized lungs, NH_3 has an action equal to its action on untreated lungs. Thus, in large doses, NH_3 can still act after atropine. It is thought that the broncho-constrictor effect of NH_3 is due to a twofold action: (1) associated with the release of acetylcholine and (2) dependent upon a direct effect on the bronchial muscle. This interpretation is not inconsistent with the view that NH_4^+ ions have properties analogous to K^+ ions as has already been indicated by Hermann, Jourdain, Morin & Vial (1938 *a*, *b*, *c*).

REFERENCES

Hermann, H. F., Jourdain, G., Morin, G. & Vial, J. (1938*a*). *C.R. Soc. Biol., Paris*, **127**, 613.

Hermann, H. F., Jourdain, G., Morin, G. & Vial, J. (1938*b*). *C.R. Soc. Biol., Paris*, **128**, 676.

Hermann, H. F., Jourdain, G., Morin, G. & Vial, J. (1938*c*). *C.R. Soc. Biol., Paris*, **129**, 595.

Coronary vein catheterization in man. By G. J. Aitken and J. C. Eaton. *Departments of Cardiology and Biochemistry, the Royal Infirmary, Glasgow*

In cardiac catheterization to determine cardiac abnormalities, entry of the catheter into the middle coronary vein enabled a sample of blood to be obtained from this vessel. Location of the catheter was shown by (1) fluoroscopy, the catheter lying along the lower cardiac border, the tip pointing upwards; (2) a mean B.P. of 5·3 mm. Hg compared with 2·2 mm. in right auricle and 65 mm. in right ventricle; (3) blood oxygen content of 7·1 ml. O_2 per 100 ml. compared with 19·8 ml. in right auricle and 12·4 ml. in median basilic vein; (4) impossibility of pushing the catheter further. (The high oxygen content of right auricular blood was due to the septal defect.)

An oxygen tension below that of a limb vein is to be expected in blood from a muscular organ at work compared with blood from a resting limb. This confirms the observations of Bing *et al.* (1947) and Dexter & Sosman (1947).

REFERENCES

Bing, R. J. *et al.* (1947). *Proc. Soc. exp. Biol. med.* **66**, 239.
Dexter, L. & Sosman, M. C. (1947). *Radiology*, **48**, 441.

Changes in arterial blood pressure caused by electrical stimulation of the sinus nerve in cats. By W. W. Douglas, I. R. Innes and H. W. Kosterlitz. *Physiology Department, University of Aberdeen*

In cats anaesthetized with nembutal or urethane, with both vagi cut, electrical stimulation of the sinus nerve caused a rise or fall in blood pressure. With constant frequency the response varied with the intensity and pulse duration of the stimulus. Supraliminal stimulation gave a feeble depressor effect in some animals, but a powerful pressor response was obtained on increasing the intensity. In others this fall on weak stimulation was not obtained, the first response being a rise, the pressor effect being more pronounced with pulses of short duration. Further increasing the intensity in many instances caused the pressor response to diminish and give way to a fall. The depressor effect was more readily obtained with long pulses. Often a rise was converted to a fall by increasing the pulse duration alone.

Euler, Liljestrand & Zotterman (1939, 1941) found small chemosensory and large and small barosensory fibres in the sinus nerve.

REFERENCES

Euler, U. S., Liljestrand, G. & Zotterman, Y. (1939). *Arch. Skand. Physiol.* **83**, 132.
Euler, U. S., Liljestrand, G. & Zotterman, Y. (1941). *Acta physiol. Scand.* **2**, 1.

Changes in the liver during pregnancy and lactation. By Rosa M. Campbell and H. W. Kosterlitz. *Physiology Department, University of Aberdeen*

A measure of nuclear material was obtained by determining deoxyribonucleic acid and of cytoplasmic material by determining protein, phospholipin and ribonucleic acid. During the second week of pregnancy in rats, an increase in the turn-over (P^{32}) and the amount of nuclear material was found with a corresponding increase of cytoplasm. During the third week there was, in addition, a considerable increase in the ribonucleic acid content, which was independent of changes in protein and phospholipin, and still occurred after removal of the foetuses as long as the placentae were left intact. An increase in lipoid P turn-over was found only when both foetuses and placentae were intact. During the first week of lactation, both deoxyribonucleic and ribonucleic acids returned to almost normal values. During the second week the ratio of cytoplasmic to nuclear material rose markedly, probably due to an increase in dietary protein intake.

On the return of sudomotor and vasomotor reflexes to the sympathectomized hand. By H. Barcroft and G. T. C. Hamilton

Sympathectomy of the arm leaves a wide gap between the fibres (preganglionic section, Smithwick (1940)). Yet Simmons & Sheehan (1939), Haxton (1947) and Barcroft & Hamilton (1948) have shown that sympathetic function may, to some extent, be regained. Further confirmation has now been obtained from the examination of sixteen limbs 1–6 months after operation, and again 1–1½ years later. When first examined, vasomotor and sudomotor reflexes were absent; all sympathectomies had been complete. The second examination, however, showed the reappearance of the reflexes in some limbs. Since clinical improvement in the vasospastic attacks persisted, the new nerve path was probably not so good as the original.

Lee (1929) has reviewed the extensive literature on the remarkable powers of regeneration of sympathetic fibres in animals.

REFERENCES

Barcroft, H. & Hamilton, G. T. C. (1948). *Lancet*, 1, 441.
Haxton, H. A. (1947). *Brit. J. Surg.* 35, 69.
Lee, F. C. (1929). *Physiol. Rev.* 9, 575.
Simmons, H. T. & Sheehan, D. (1939). *Brit. J. Surg.* 27, 234.
Smithwick, R. H. (1940). *Ann. Surg.* 112, 1085.

Elastic properties of normal and rachitic rat femora. By G. H. Bell, J. W. Chambers and J. B. de V. Weir. *Department of Physiology and Biochemistry, University College, Dundee; University of St Andrews and The Institute of Physiology, Glasgow University*

The elastic properties of the femora of rats on normal and rachitogenic diets have been investigated. When a femur is supported at its ends and loaded at the centre it sags and from this sag and the dimensions of the bone section Young's modulus (E) can be calculated. The rachitic bones gave values for E of about $0{\cdot}6 \times 10^6$ lb./in.^{2} whereas normal bones gave a value of $1{\cdot}6 \times 10^6$ lb./in.^{2}. Although there is considerable variation in the stress at the elastic limit in normal and rachitic bones yet the strain is remarkably constant at about 1·5%. The breaking stress of bone is very nearly that of cast iron, but its E value is about ten times less. The greater flexibility of bone has obvious biological advantages.

Heat injury and new vessel formation in the rabbit's cornea. By F. W. Campbell and I. C. Michaelson (introduced by R. C. Garry). *Institute of Physiology, Glasgow University*

Heat burns of constant intensity and size were placed on the corneae of adult rabbits at varying distances from the limbus. Each burn was repeated daily for 10–14 days. The injuries were made under local 'Pontocain' anaesthesia. Such injury evoked a new growth of vessels into the superficial layers of the corneae only when the lesion was within a certain critical distance of the limbus. When new-vessel growth occurred it was studied by mounting the corneae, after Indian ink injection, in glycerine.

The vascular response took the form of an isosceles triangle. The equal sides were of almost constant length in each triangle and were independent of the site of the effective lesion.

The results suggest liberation of a substance at the site of the lesion responsible for the vascular new growth.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

25 *September* 1948

Histamine test for gastric secretory function in cats. By R. E. Davies and D. H. Smyth. *Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry and Department of Physiology, University of Sheffield*

For experiments on gastric secretion in cats it is of great value to select animals with a large capacity for HCl secretion, and this can be conveniently done by a histamine test under light cyclopropane anaesthesia. About 20 min. after a subcutaneous injection of histamine (0·5 mg. histamine hydrochloride/kg.) the cat is anaesthetized with a mixture of cyclopropane and oxygen by means of a mask, a wooden gag is inserted in the mouth and securely tied, and a tube is passed into the stomach through a hole in the gag. The anaesthetic mask is then replaced and the stomach tube led out through a special opening so that gastric samples can be obtained while the cyclopropane is being administered. The mask is provided with a CO_2 absorber and rubber bag so that rebreathing occurs, but as there is always some leakage between the mask and the face a small constant supply of cyclopropane and oxygen is maintained. At 15 min. intervals the stomach is washed out with four lots of 20 ml. of 0·9% NaCl at about 40° C. and the washings are titrated with 0·1 N-NaOH. The method is not always strictly quantitative as sometimes all the saline may not be recovered, but the first two or three 15 min. samples show whether the animal gives a good response to histamine, and no attempt is made to continue the experiment until gastric secretion ceases. In a series of thirty-seven cats the range of the maximum rate of secretion varied from 0·0 to 12·8 ml. 0·1 N-acid/15 min.

A respiration pump for use with closed air circuit. By A. A. Glynn and D. H. Smyth. *Department of Physiology, University of Sheffield*

The use of artificial respiration with a closed circuit for measuring oxygen consumption demands a pump absolutely free from leakages, and with the ordinary mechanical pump of the 'Ideal' type this is difficult to ensure. The following device (Fig. 1) used with an 'Ideal' pump ensures a completely closed circuit, as the circulating air does not come into contact with the pump.

The pump is connected with the point *A* and on inflation depresses the water level in *B* and the mercury level in *C*. As a result the water level in *D* rises and also the mercury levels in *E* and *F*, thus closing off the tubes dipping into the mercury. The water level rising in *D* now inflates the animal's lungs, since the other exits are closed by the mercury. When inflation ceases, the level of mercury in *E* and *F* suddenly falls, opening the tubes and allowing the lungs

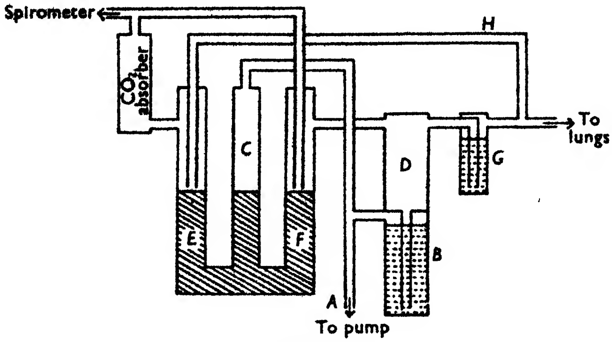


Fig. 1.

to empty by their own elasticity. The presence of the water valve at *G* causes the expired air to travel by the tube *H* to the CO_2 absorber. It also causes the tube *D* to be filled with air from the absorber and hence maintains an air circulation.

The apparatus is also useful when a closed circuit is used for cyclopropane anaesthesia as the cyclopropane is soluble in the oil lubricating the metal parts of the pump.

An oxygenator for use with closed circuit for measurement of oxygen consumption. By D. H. Smyth. *Department of Physiology, University of Sheffield*

The oxygenator (Fig. 1) is intended for use in perfusion experiments where it is desired to record oxygen consumption.

The special features of the oxygenator are:

(1) Any desired level of CO_2 tension can be maintained in the oxygenator, while at the same time the O_2 consumption is estimated with a spirometer after CO_2 absorption.

(2) An even spread of the blood is ensured by constant rotation of the oxygenator.

The glass oxygenator and reservoir *A* rotates to and fro about its long axis making about one complete turn in each direction. A pump connected with tube *B* causes a rhythmic rise and fall of the water level in tube *C*. This causes

a tidal air to pass in and out of the oxygenator by the tube *D* which is connected through a pair of water valves *E* to maintain a circulation of air through a closed circuit containing a spirometer and CO_2 absorber. The concentration of CO_2 in the oxygenator depends on the 'pulmonary ventilation' which can

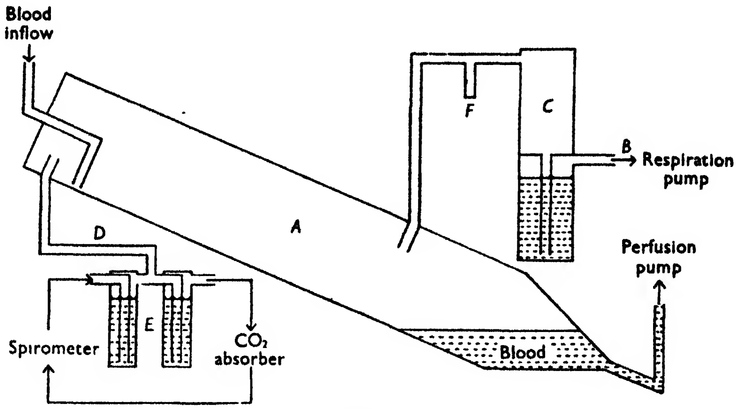


Fig. 1.

be adjusted by altering either the rate or stroke of the pump. Samples of air are withdrawn by tube *F* for analysis and the pulmonary ventilation adjusted to give the required CO_2 concentration. A central core (not shown in the figure) inside the oxygenator increases the surface for exposure of the blood to oxygen and also diminishes the volume of gas in the oxygenator.

Apparatus for automatic maintenance of body temperature in anaesthetized animals. By D. H. Smyth. *Department of Physiology, University of Sheffield*

The maintenance of normal body temperature in anaesthetized animals during acute experiments demands considerable attention in the absence of an automatic apparatus for the purpose. Many arrangements have been devised for automatic temperature control, but for routine experiments there is still a need for a simple apparatus easily operated and preferably obtainable commercially. The following apparatus, which has been in use for some time in metabolism experiments, fulfils this need.

The principle is to regulate the heating of the operating table by two separate controls, providing a coarse and fine adjustment. The former, which can be altered over a wide range, is set at such a level as to overheat the table by a small amount. The fine adjustment is a thermoregulator in the animal's rectum to prevent a rise in temperature. The latter instrument alone is not

sufficient, as in the absence of the coarse adjustment there is too much overswing past the mean temperature both in warming and cooling.

Instruments which have been found very suitable for these purposes are produced commercially by Sunvic Controls Ltd. The coarse adjustment is a 'No-Loss' Energy Regulator. This contains a bimetal strip with a heater winding, and on a passage of a current for a certain time the bimetal operates a switch and breaks the circuit. After a certain period of cooling the circuit is made again, and the cycle repeated. By means of a control screw the ratio of 'on' and 'off' times can be altered so that any desired fraction of the heating capacity of the operating table can be utilized. Experience shows what amount of heating is required for each type of animal to cause a very slow rise in body temperature. For the fine control an adjustable thermostat is used which responds to a change of 0·1° C. It consists of a bimetal helix mounted on a metal stem. The helix is of such a size as to fit easily into the rectum of a cat. The only modification required of the commercial article is to provide a metal cap to prevent contamination with the faeces. This lessens the sensitivity, but still leaves the instrument sufficiently sensitive for physiological purposes.

The two instruments can be used directly in series without any relay on a.c. mains, and will maintain the temperature of the cat within 1° C.

Haemolysis with Paludrine and its acceleration in the haemocytometer chamber. By G. Wiseman. *Department of Physiology, University of Sheffield*

Preliminary experiments on bacterial sensitivity to Paludrine (Steingold, 1948) showed haemolysis of human erythrocytes with high concentrations of Paludrine.

In order to examine this haemolysis, solutions of pure Paludrine hydrochloride (20–200 mg. %) are made up in 0·94% sodium chloride having a pH between 4·6 and 6·0. Fresh human erythrocytes are diluted 1 in 200 with these solutions in haemocytometer pipettes and erythrocyte counts made at intervals. With a concentration of 200 mg. % Paludrine successive counts show almost complete disappearance of erythrocytes in 80–100 min., and with smaller concentrations a slower rate of disappearance is observed. However, it is observed that if the solution is left in the haemocytometer chamber haemolysis occurs much more rapidly, being complete in about 10–15 min. with concentration of 200 mg. % Paludrine. The rate of haemolysis is not affected by light.

This rapid haemolysis can also be observed microscopically in capillary tubes. The rate is increased when finer tubes are used and is retarded if the capillaries

are coated with wax. If the contents of the capillary tubes are centrifuged the presence of haemoglobin in the supernatant fluid shows that true haemolysis has occurred.

Careful observation of the rapid haemolysis shows a definite series of morphological changes and the erythrocytes can be seen to disappear suddenly, leaving a 'ghost' which disappears after a further interval.

Using the 0·94% saline as control, erythrocytes can be kept in the pipette or counting chamber for over 30 hr. without appreciable haemolysis. The pipettes and counting chambers are kept in a water-saturated atmosphere between observations.

These experiments suggest that the rate of haemolysis due to Paludrine is increased on increasing the area of contact of the glass surface. It has been suggested by Ponder (1944) that, in general, contact with the glass surface retards the rate of haemolysis.

I am indebted to I.C.I. for samples of Paludrine hydrochloride and Paludrine lactate.

REFERENCES

Ponder, E. (1944). *Medical Physics*, p. 606. Chicago: Year Book Publishers.
Steingold, L. (1948). Personal communication.

Central action of tetramethyl- and tetraethyl-ammonium salts and of erythroidines.

By S. SALAMA (introduced by SAMSON WRIGHT). *Department of Physiology, Middlesex Hospital Medical School, London*

In a previous communication I reported that certain of the natural curare alkaloids are central excitants, with the exception of the bebeerines which are devoid of central action. The depression of reflexes produced by intraventricular or intrathecal injection of erythroidines is due to a peripheral action.

Tetramethyl-ammonium and tetraethyl-ammonium salts, however, behave anomalously. Intraventricular and intrathecal injections of tetramethyl-ammonium iodide inhibit both the knee-jerk and flexor reflex by a central action. Intravenous injections of tetraethyl-ammonium bromide inhibit the knee-jerk and augment the flexor reflex. On intraventricular or intrathecal injection, however, it increases the tone of extensor muscles, and causes spontaneous contractions in them, but does not affect the flexor reflex. These effects are similar to those obtained with nicotine.

The role of carbon dioxide in the secretion of hydrogen and bicarbonate ions.

By R. E. Davies (introduced by D. H. Smyth). *Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield*

For each H^+ ion secreted, oxyntic cells produce 1 OH^- ion. To neutralize this 1 CO_2 molecule is hydrated to produce H_2CO_3 which reacts with the OH^-, forming H_2O and HCO_3^- (Davies, 1948). This hydration requires carbonic anhydrase within oxyntic cells (Davies & Roughton, 1948). In actively acid-secreting stomachs CO_2 uptake exceeds O_2 uptake and the CO_2 available from metabolism in the stomach or from that dissolved in the arterial blood. The extra CO_2, made available by blood carbonic anhydrase, comes from blood HCO_3^-.

A similar process could account for HCO_3^- production in pancreas and duodenum, and for HCO_3^- reabsorption in kidney. H^+ ions secreted into the blood or tubular fluid liberate CO_2 which diffuses into the cell, neutralizes the OH^- ions formed concomitantly with the H^+ ions and is passed into the secretory ducts or blood as HCO_3^- ions. Both dehydration and hydration of CO_2 require carbonic anhydrase. This theory resembles that of Pitts (1945), but allots a different role to carbonic anhydrase in HCO_3^- transport.

REFERENCES

Davies, R. E. (1948). *Biochem. J.* **42**, 609.
Davies, R. E. & Roughton, F. J. W. (1948). *Biochem. J.* **42**, 618.
Pitts, R. F. (1945). *Science*, **102**, 49, 81.

Electrical stimulation of nerve fibres from the chemoceptors and baroceptors of the carotid in the dog.

By E. Neil, C. R. M. Redwood and A. Schweitzer. *Departments of Physiology, School of Medicine, Leeds, and University College, London*

Owing to the fact that the nerve fibres from the carotid sinus are joined by those from the carotid body within a few millimetres of their origin, separate electrical stimulation of these two types of fibre is usually impossible. It is of interest therefore to report the results of an experiment on a dog under chloralose anaesthesia, in which the carotid body fibres were found to run separately from the sinus fibres up to the point of junction with the glossopharyngeal nerve.

Rectangular wave stimulation of the carotid body fibres (2 V. 50 cyc./sec.) with pulse duration of 10 msec. produced marked hyperpnoea with little effect on the arterial blood pressure. Shorter pulse durations did not elicit hyperpnoea.

Stimulation of the carotid sinus nerve fibres caused a fall of blood pressure and an inhibition of respiration with pulse durations of 0·5–1·0 msec. Longer pulse durations were ineffective.

Pathways of afferent impulses from the chemoceptors of the aortic body in the cat. By E. NEIL, C. R. M. REDWOOD and A. SCHWEITZER. *Departments of Physiology, School of Medicine, Leeds, and University College, London*

Cats were anaesthetized with chloralose (0·08 g./kg. body weight). Both carotid sinuses were denervated. The aortic nerves were isolated from the vagi in the neck. Intraventricular cannulation was effected (Comroe, 1939). Lobeline (0·2 mg.) and nicotine (0·04–0·08 mg.) were injected intraventricularly to stimulate the aortic chemoceptors. Respiratory and blood pressure responses were recorded.

After section of both vagi about half the original hyperpnoeic response to injection still remained. This response was mediated via the aortic nerves, for sectioning these nerves abolished it.

Similar experiments, in which the vagi and aortic nerves were cut separately on each side, showed that a preponderance of the afferent fibres from the aortic body pass on the right side via the vagus and aortic nerve.

REFERENCE

Comroe, J. H. (1939). *Amer. J. Physiol.* **127**, 176.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

23 *October* 1948

A method for the explantation of the right kidney in the dog.

By MARY F. LOCKETT. *Pharmacology Laboratory, University College, London*

Rhoads (1934) published a method for the explantation of the left kidney in the dog which rendered the left renal vein readily accessible. He discussed the renal nerve supply, and showed how such a preparation could be made with intact innervation. Lockett, O'Connor & Verney (1942) described a method by which access was gained to the left kidney; the pedicle was exposed, and a renal artery loop was made. If so desired the kidney was denervated at the time of the final operation.

The right kidney of the dog lies proximal to the left, and is normally completely covered by the lower ribs when approached from the posterolateral aspect; the hilum is not infrequently opposite the tenth intercostal space. The only problem which presents itself in a right renal explantation in the dog is the high position of the right kidney. This difficulty has been surmounted by the subperiosteal resection of the lower four ribs, coupled with a proximal transplantation of the posterior part of the diaphragm. The height to which the diaphragm must be moved varies with individual animals, and is determined by direct observation at the time of operation.

By the method described, the right kidney can be explanted and either a right renal artery loop made, or the kidney pedicle can be stitched beneath a skin flap, giving ready access to the renal vein.

REFERENCES

Lockett, M. F., O'Connor, W. J. & Verney, E. B. (1942). *Quart. J. exp. Physiol.* **31**, 333.

Rhoads, C. P. (1934). *Amer. J. Physiol.* **109**, 324.

Apparatus for the fractional sampling of a single expiration.

By G. H. ARMITAGE, W. MELVILLE ARNOTT and A. C. PINCOCK. *Department of Medicine, Queen Elizabeth Hospital, University of Birmingham*

There are several problems in both physiological and pathological respiratory function which necessitate samples of an expiration taken in such a way as to enable the construction of a complete balance-sheet of the expired gases.

Krogh & Lindhard (1914), Nielsen & Sonne (1932) and Roelsen (1938) have devised methods for doing this, and our apparatus is in principle the same as that used by these authors, differing only in technical details.

The subject expires through a mild steel tube (non-ferrous metals are unsuitable because of their reactivity with mercury) into a Benedict Roth Spirometer which records on a rapidly travelling drum (Fig. 1). Four inches from the mouthpiece the steel tube has a collar through which is drilled a ⅛ in.

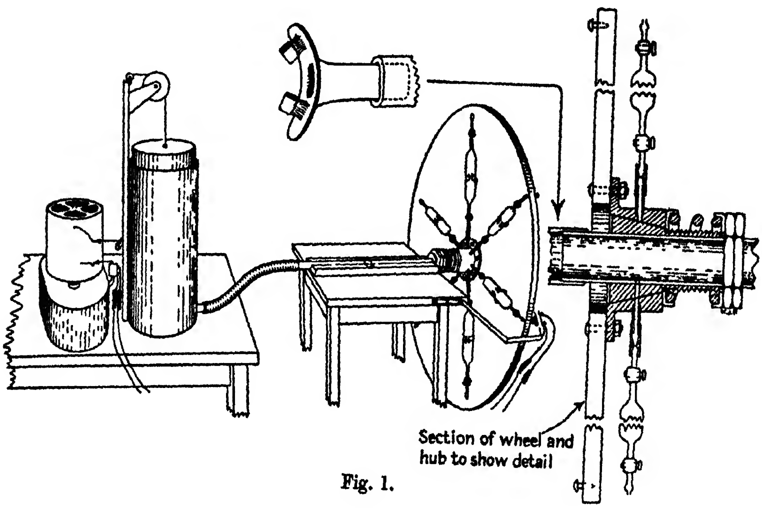


Fig. 1.

wide hole. This collar is cone-shaped, and on it rotates the steel hub of a 28 in. wide plywood wheel which carries at intervals of 60° evacuated gas sampling tubes, each of which is attached to a ⅛ in. wide hole in the hub. During one rotation of the wheel each hole passes over the single port in the collar, thus establishing brief communication with the lumen of the steel tube. The bearing surfaces are accurately ground, well greased and spring loaded so as to ensure the maintenance of a vacuum in the sampling tubes.

The filling of each sampling tube is indicated on the drum by a signal—in vertical alinement with the pen of the spirometer—energized by contacts, closed by studs on the circumference of the wheel.

We are indebted to Prof. G. F. Mucklow of the Department of Mechanical Engineering, in whose Department the steel portions of the apparatus were fashioned.

REFERENCES

Krogh, A. & Lindhard, J. (1914). *J. Physiol.* **47**, 431.
Nielsen, E. & Sonne, C. (1932). *Acta med. Scand.* Suppl. **50**, 33.
Roelsen, E. (1938). *Acta med. Scand.* **95**, 452.

Arrangement for obtaining X-ray diffraction patterns of irrigated mammalian tissues at controlled temperatures. By J. Elkes and F. W. J. Teale. *Department of Pharmacology, University of Birmingham*

A long-spacing X-ray diffraction camera for use with irrigated frog tissues has been previously demonstrated. Fig. 1 illustrates the essential features of an arrangement for obtaining X-ray diffraction patterns of small pieces of mammalian tissue at controlled temperatures. The specimen is mounted in a hydrogen-filled heated aluminium camera. Hydrogen and irrigation fluid are preheated in a thermostat, and specimen temperatures during exposure are continuously checked by a recording thermocouple.

The cylindrical camera measures 20 cm. in diameter, and is fitted with a removable base and lid which are sealed with rubber rings *Rr* during exposure. The adjustable beam collimator *C* (whether of the slit or pinhole type) is in line with a small optical bench carrying specimen holder *Sh*, stop *St*,

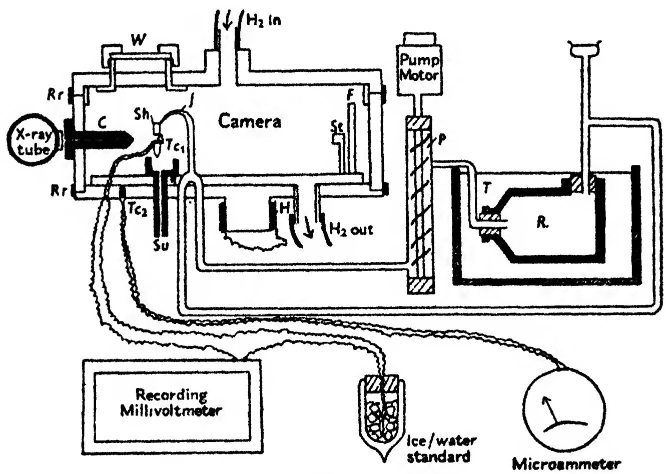


Fig. 1.

and film-frame *F*. The specimen holder, which is controlled by centring screws, consists of a small plastic block perforated by two holes measuring 5 mm. These take the specimen and standard calibration material respectively. An adjustable arm (not shown) carries a fine glass jet *J* which delivers a steady stream of preheated oxygenated Ringer solution from the reservoir *R* placed in a thermostat *T*. The rate of irrigation is controlled by the work of the non-metallic screw pump *P*, and a screw clip on the jet. Waste Ringer flows down a sump *Su*. Hydrogen filling the camera is preheated by passing through copper coils immersed in the thermostat, and enters by way of an inlet situated next to the observation window *W*. The hydrogen outlet is in the camera base. The latter is warmed by a 60 W. heater *H*, controlled by a rheostat.

A copper-constantan thermocouple Tc_1, incorporated in the specimen holder and connected to a continuously recording millivoltmeter records the specimen temperature during the experiment. Another thermocouple Tc_2 measures the camera temperature.

Temperatures up to 50° have been steadily maintained for between 2 and 6 hr., and the effect of changes of hydration and temperature on the diffraction pattern of rat adipose tissue has been studied.

Cytological changes in the columnar epithelial cells of the rat's small intestine during fat absorption.

By T. D. WILLIAMS (introduced by R. A. GREGORY). *Physiological Laboratory, University of Liverpool*

The changes in the columnar cells of the small intestine during fat absorption have been followed by feeding rats (previously starved for 18 hr.) with 0·5 ml. of olive oil (free of fatty acid) by stomach tube. At ½, 1, 2, 3 and 4½ hr. later the rats were killed and the intestines fixed by distension with formol-saline or formol-calcium (Baker, 1946).

Frozen sections from different levels are stained with Sudan IV, Sudan black, and by the acid haematin method for phospholipid (Baker, 1946). The first evidence of fat absorption is the deposition of phospholipid (acid-haematin and Sudan black) between the nucleus and the free border, except in a narrow zone immediately beneath the free border. This zone, which remains clear at all times, contains a high concentration of phosphatase (Emmel, 1945) and is presumably the site of phosphorylation. The next stage appears to be enlargement of the vacuoles of the Golgi complex and the deposition on them of phospholipid. This is then transformed into fat or fatty acid (Sudan IV) possibly by the phosphatase-like enzyme demonstrated in the Golgi complex of the intestinal cells by Deane & Dempsey (1946).

As the fat thus formed increases, it appears to break away from the Golgi complex, the cytoplasm becoming filled with fine globules (Sudan IV), which present an appearance similar to that described by Frazer (1943) during the absorption of fatty acid. These small globules apparently coalesce and pass into the core of the villus. No differences in the above picture are apparent at different levels of the intestine, which is unexpected in view of Frazer's evidence (1947) that triglycerides are absorbed unsplit from the upper part of the intestine and as fatty acids at lower levels.

REFERENCES

Baker, J. R. (1946). *Quart. J. micr. Sci.* **87**, 446.
Deane, H. W. & Dempsey, E. W. (1946). *J. cell. comp. Physiol.* **27**, 159.
Emmel, V. M. (1945). *Anat. Rec.* **91**, 39.
Frazer, A. C. (1943). *J. Physiol.* **102**, 306.
Frazer, A. C. (1947). Communication XVII Internat. Physiol. Cong. Oxford.

The induction of the 'deficiency pattern' in intestinal radiographs of normal human subjects. By A. C. Frazer, J. M. French and M. D. Thompson. *Department of Pharmacology, University of Birmingham*

The normal radiographic pattern of the small intestine consists of more or less evenly spread barium showing up the feathery pattern of the intestinal mucosa. In the so-called 'deficiency pattern', the barium is seen in isolated masses without any appreciable mucosal pattern. This 'deficiency pattern' is particularly observed in cases associated with steatorrhoea, but it can also be seen in apparently normal subjects from time to time and it is the usual pattern in young babies. Stannus (1942) suggested that the radiographic changes might be due to products of fat digestion, but this has been denied by other workers. Golden (1945) ascribed the 'deficiency pattern' to a neuro-muscular disorder consequent upon deficiency of B vitamins.

The object of this demonstration is to show experimental radiographic studies and other investigations which indicate the nature and aetiology of the 'deficiency pattern'.

The first group of experiments shows the induction of a radiographic pattern indistinguishable from the 'deficiency pattern' in normal human subjects. The barium sulphate suspension was injected intraduodenally with and without the addition of various substances. It will be seen that hypertonic solutions, fatty acids and lactic acid induced the 'deficiency pattern'.

The second group of experiments shows that unhydrolysed fat does not affect the intestinal pattern. Hydrolysis of the fat results in the development of a characteristic 'deficiency pattern' and this action can be reversed or prevented by the administration of calcium salts.

The third group of experiments indicates that the pattern is probably due to flocculation of the barium suspension rather than neuro-muscular changes in the intestinal wall. They also suggest the possible importance of mucin as an aetiological factor in the development of the 'deficiency pattern'.

It is concluded that the so-called 'deficiency pattern' can be induced in human subjects by the administration of the products of lipolysis; it is not a deficiency condition; its development may depend upon the presence of mucin.

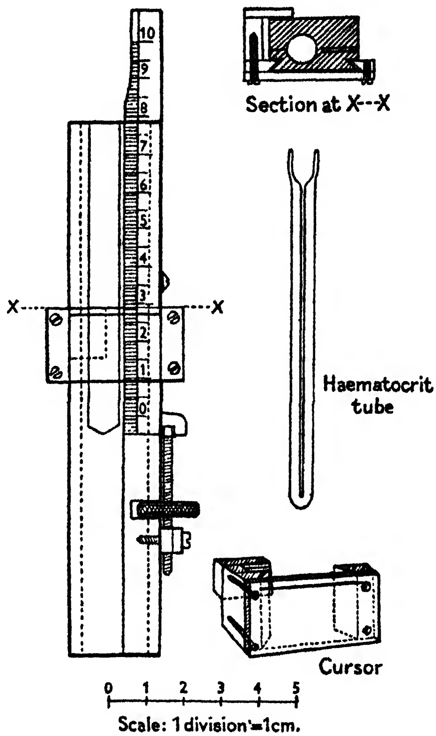
REFERENCES

Golden, R. (1945). *Radiologic Examination of the Small Intestine*. Philadelphia: Lippincott.
Stannus, H. S. (1942). *Trans. R. Soc. trop. Med. Hyg.* **36**, 123.

An instrument for accurate reading of an improved Meyerstein haematocrit tube. By H. P. Gilding, W. Meyerstein and M. E. Nutt.

Department of Physiology, University of Birmingham

The instrument is made of Perspex. It consists of a holder for the haematocrit tube with a movable scale alongside. A cursor with lines engraved on front and back avoids errors due to parallax. The scale lies in a slot and consists of a piece of white celluloid ruler, graduated in 0·5 mm., attached at its lower end to the adjustment screw, and is kept vertical by a retaining spring.



The Meyerstein tubes are made from Chance's precision glass capillary tubing, guaranteed internal diameter $0{\cdot}8 \pm 0{\cdot}01$ mm. The lower end of the tube is sealed with a glass plug (drawn out from the same sample of glass) in such a way that the end of the capillary is flat and at right angles to its length; the top is expanded into a cup of 0·4 c.c. capacity. Evaporation is prevented by delivering about 30 cu.mm. of blood under liquid paraffin in the cup, using a pipette made from similar capillary tubing to that of the haematocrit.

After centrifuging, the haematocrit tube is placed in the holder and the zero of the movable scale adjusted to coincide with the bottom of the column of blood. Height of cells and total height are read off and the percentage cells calculated.

When statistically analysed, results obtained by the Meyerstein tubes showed no significant difference from those obtained by Wintrobe tubes spun along with them. Use of liquid paraffin is justified, since results on Wintrobe tubes, with and without the use of paraffin, showed no significant difference. (Loss by evaporation from Wintrobe tubes may be as much as 5% of the total volume. Hence the necessity for reading the height of the column of blood before centrifuging.)

When spun at 5400 r.p.m. in a centrifuge, radius 15 cm. ($F \equiv 4860 \times$ gravity

at the periphery) the standard deviation of a series of forty-five determinations using fifteen different Meyerstein tubes was 0·245. Using wooden blocks suitably drilled it is possible to centrifuge twenty-four or more tubes at once.

REFERENCE

Meyerstein, W. (1942). *J. Physiol.* **101**, 5*P*.

The excretion of creatinine in the dog.

By J. A. Barclay. *Department of Physiology, University of Birmingham*

In seventy-two observations on five anaesthetized dogs the clearance ratio of inulin/endogenous creatinine was found to average 3. Raising the plasma level of creatinine by slow intravenous drip resulted in an abrupt fall to a ratio of 1. It is suggested that creatinine in the dog has a reabsorption tubular mass (*Tm*). The value of this is so small as to make its exact determination difficult—it is probably not greater than 0·5 mg./min.

The reappearance of co-ordinated movements of the hand after lesions in the hand area of the motor cortex of the rhesus monkey.

By P. Glees and J. Cole. *University Laboratory of Physiology, Oxford*

Experiments on the distribution of cortico-spinal fibres from area 4 show that no definite area within the internal capsule is assigned to a particular subdivision of area 4; on the contrary, fibres from all subdivisions intermingle in their descending course. Small lesions in the hand area cause degeneration not only at cervical levels, but down to the lumbar region, suggesting that subdivisions of area 4 connect with several spinal levels. The reappearance of a trained motor act after unilateral and bilateral lesions of area 4 has been studied and the findings of Leyton & Sherrington (1917) and Kennard (1936, 1942) confirmed. In addition, we found no interference with trained co-ordinated movements of the previously paralysed limb. Re-exposing area 4 after extirpation of the arm area, mapped out by stimulation, revealed that all hand responses could be elicited from areas adjacent to the lesion in contrast to the observations of Grünbaum & Sherrington (1903) on the chimpanzee. Undercutting these areas caused recurrence of paralysis in the previously recovered arm.

The reappearance of a motor pattern may be explained by the plurisegmental connexions of area 4 as described above. This would also explain the results of stimulation of the foetal and infant's motor cortex by Hines (1943).

REFERENCES

Grünbaum, A. S. F. & Sherrington, C. S. (1903). *Proc. Roy. Soc.* **72**, 152.
Hines, M. (1943). *Biol. Rev.* **18**, 1.
Kennard, M. (1936). *Amer. J. Physiol.* **115**, 138.
Kennard, M. (1942). *Arch. Neurol. Psychiat., Chicago*, **48**, 227.
Leyton, A. S. F. & Sherrington, C. S. (1917). *J. exp. Physiol.* **11**, 135.

Blood flow in the circle of Willis. By D. A. McDONALD and J. M. POTTER (introduced by K. J. FRANKLIN). *Department of Physiology, St Bartholomew's Hospital Medical College*

To study the distribution of blood from individual cerebral arteries we have adapted the rapid coagulation technique of Franklin & Amoroso (1948). Using rabbits, we introduced a coagulant (thrombin or Russell's viper venom) plus Evans's blue dye into the internal carotid artery by retrograde injection of the external carotid; the vertebral was similarly injected via the subclavian artery. By the use of suitable precautions, the volume flows in the arteries studied were left unchanged.

Results show that each artery supplies a separate territory of the brain, that there is a distinct demarcation in the posterior communicating artery between opposing streams of blood, and that even in the basilar artery the flow from each vertebral can remain distinct for a varying distance.

If the normal balance of pressure is altered, the circle of Willis becomes a *functioning* anastomosis and the blood distribution changes.

REFERENCE

Franklin, K. J. & Amoroso, E. C. (1948). Oral communication to the Physiological Society 20 March. Unpublished.

The action of curarizing substances on respiration in the cat. By W. D. M. PATON and E. J. ZAIMIS. *National Institute for Medical Research, Hampstead, N.W. 3*

In cats anaesthetized with chloralose, the respiratory depression associated with a given paralysis of tibialis is much greater with D-tubocurarine (D.T.C.) than with bistrimethylammonium decane (C10, Paton & Zaimis, 1948). D.T.C. causes respiratory depression before significantly affecting tibialis twitch; but tibialis can be 95% paralysed with C10 before the respiration is affected. D.T.C. in the doses used does not alter the respiratory discharge down the phrenic nerve, unless asphyxia occurs, and does not cause bronchoconstriction. Its respiratory action is largely due to its strong depression of a tetanus

(20 per sec.) in doses which have little effect on a twitch; with C10, however, the tetanus is well sustained. In addition, D.T.C. is more and C10 less active on red muscle (soleus, diaphragm) than on white muscle (tibialis). The sparing of respiration shown by C10 and related compounds is roughly in proportion to their anticholinesterase activity, but the selective respiratory depression of D.T.C. is not prevented by eserine or prostigmine.

REFERENCE

Paton, W. D. M. & Zaimis, E. J. (1948). *Nature, Lond.*, **161**, 718.

PROCEEDINGS
OF THE
PHYSIOLOGICAL SOCIETY
17–18 *December* 1948

Plethysmograph for studying respiration in the new-born infant. By K. W. Cross. *Department of Physiology, St Mary's Hospital Medical School, London*

Automatic integrator for volume recorders. By P. W. Roberts and W. F. Widdas. *Departments of Physics and Physiology, St Mary's Hospital Medical School, London*

The problem was to find a method for the continual summation of the excursions made by the float of the plethysmographic volume recorder.

A mechanical ratchet device drawing its power from the writing lever caused such resistance that the excursions were unreliable. A method not taking its power from the recorder was essential.

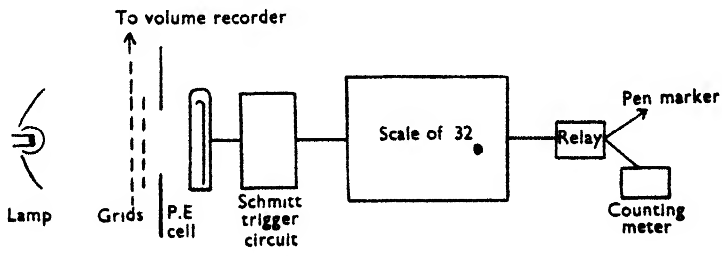


Fig. 1. Block diagram of integrator.

While a mirror causing an image of a slit to scan a grid placed before a photocell could be used in a bench experiment, there are disadvantages to its use in the confined space and mobile 'set up' of the apparatus on a ward trolley. The method to be described has the advantage of keeping the optical train short, robust and direct while facilitating the attainment of adequate light intensity on the photocell with only a 6 V. torch bulb.

Essentially, the apparatus consists of: (1) a photocell and optical system designed to interrupt a beam of light many times for a small movement of the plethysmograph; and (2) an electronic circuit which receives and counts the

electrical impulses from the photocell and operates a pen which registers the volume change on the paper trace.

Fig. 1 shows a diagrammatic arrangement of the apparatus.

The interrupter unit

Light from a 6 V. bulb passes through two thin photographic plates each having horizontal dark lines alternating with clear spaces. Relative movement of one grid over the other will alternately transmit and interrupt the light beam according to whether the opaque lines of one grid fall in position behind the opaque or transparent lines of the other grid. As there are 60 dark lines per cm. a complete cycle of change of light intensity will occur every $\frac{1}{6}$ mm. and this is the information which the photocell passes on to the electronic circuit.

The movable grid travels in a machined brass guide which is mounted on the side of the volume recorder and which can be set vertical by adjusting screws. The fixed grid can be accurately aligned relative to the movable grid.

Although the mass of the movable grid (4·7 g.) and its counterpoise add to the inertia of the volume recorder more than a mirror system would do, extensive calibration runs have proved satisfactory and show that this small increase is not a factor of any significance over the range of frequencies concerned.

The photographic grids were supplied to our specification by Messrs Kodak Ltd.

The electronic integrator

The light impulses may attain frequencies approaching 300 per sec. and cannot therefore be handled by an electromechanical counter limited to 10 per sec. Electrical impulses from the photocell amplifier are therefore fed into a scaling circuit before operating the counter.

The photocell amplifier consists of a Schmitt trigger circuit which supplies the necessary steep-fronted pulses to operate the scaler. The Schmitt circuit is not dependent on the speed at which the light beam is interrupted but only on the event occurring.

The Scaling circuit is a 'scale of 32' of conventional design. The output closes a relay which in turn operates both a pen and an electromechanical Post Office counter.

It is felt that such equipment is not restricted to the solution of this particular problem, but may have wider applications where irregular movements are to be integrated.

Demonstration of the 'muscle pump' in the human leg. By H. BARCROFT and A. C. DORNHORST. *St Thomas's Hospital Medical School, London*

Changes in calf volume are followed by means of a simple plethysmograph, the foot circulation being occluded during observation by an ankle cuff.

The subject is arranged on a couch in such a way that he can depress a weighted foot-plate by a contraction of his calf muscles above. Rhythmic contraction and relaxation is performed in time with a metronome. This exercise produces regular oscillations of recorded calf volume which do not prevent progressive changes in mean volume from being detected.

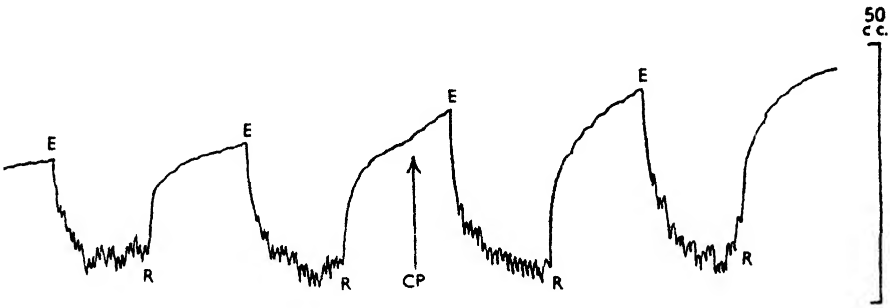


Fig. 1. *E*, pedal pressed down once a second for 10 sec. *R*, rest for 10 sec. *CP*, cuff just above knee inflated to 90 mm. Hg till end of recording.

When the subject begins rhythmic exercise his calf shrinks, the rate of shrinkage becoming less with successive contractions until a steady volume is reached. When he stops exercise his calf rapidly swells to regain its original resting volume.

By placing a cuff, inflated to a desired pressure, proximal to the plethysmograph, and observing its influence, if any, on these volume changes, an estimate is made of the resistance that the 'pump-out' mechanism can overcome.

It is demonstrated that in a normal subject a cuff pressure of 90–100 mm./Hg may fail to suppress the mechanism.

Simultaneous recording of spirogram and thoracogram. By H. HERXHEIMER. *University College Hospital, London*

By tracing respiration simultaneously with a spirometer and the thoracograph of Verzár (1945) the exchanged volume of air and the change of chest circumference—the costal movements—are recorded. By comparing the two an approximate estimate of the contribution of diaphragm and ribs to the respiratory exchange can be obtained. The inspiratory and expiratory limits

of the tidal air can be used on both tracings as fixed points for investigating the subdivisions of the vital capacity. If the breath is held at arbitrary intervals during the recording of these subdivisions, fractions of these can also be examined. It can be shown that the shares of diaphragm and ribs in the air exchange may change abruptly during one respiratory cycle. For instance, the last part of a maximum inspiration is carried out with much more costal movement than the corresponding first part of the following expiration. This is similar in maximum expiration.

REFERENCE

Verzár, F. (1945). *Höhenklimaforschungen Basler Physiol. Inst.* p. 77. Basel: B. Schwabe and Co.

Excretion of sodium by human erythrocytes. By M. MAIZELS. *The Department of Clinical Pathology, University College Hospital*

Erythrocytes of cold-stored blood lose K and gain Na. On incubation cells gain K and lose Na against the respective concentration gradients. To decide if both cation movements were active or one active and the other passive and compensatory, heparinized blood was cold-stored with saline. After 6 days cell contents were Na 56; K 65 m.equiv./l., changing after 20 hours' incubation to Na 20; K 88. Parallel experiments with cold-storage in LiCl solution gave cell contents: Na 10; K 81; Li 34, changing in incubation to Na 6; K 80; Li 50 (external Na 40 m.equiv./l.), erythrocytes distinguishing between cations and expelling only Na. Since with Na output limited by the preceding low cold-storage value, K uptake fails, K uptake is secondary to an Na expulsion, which *in vivo* checks inflow from the Na-rich environment. This inflow, imposed by the concentration gradient and non-penetrating cell anion would, unchecked, lead to cell rupture.

Vaso-dilatation in response to heating the skin. By K. E. COOPER and D. McK. KERSLAKE. *R.A.F. Institute of Aviation Medicine, Farnborough*

Considerable but inconclusive evidence exists (Duthie & Mackay, 1940) which suggests that generalized vaso-dilatation takes place in response to afferent nervous stimuli arising in locally heated areas of skin.

Further evidence supporting this hypothesis has been obtained by heating the chest and abdominal skin under a radiant heat cradle. The hand blood flow increased, the rise commencing within 12 sec. of switching on the lights. The time of onset of this dilatation was independent of the resting hand blood flow and of the intensity of illumination within wide limits.

Heating the front of the legs caused a smaller increase in hand blood flow, having the same time relations. This response was unaltered when the circulation to the legs was arrested.

The vaso-dilatation was found to be associated with a fall in mouth and rectal temperatures.

REFERENCE

Duthie, J. J. R. & Mackay, R. M. I. (1940). *Brain*, **63**, 295.

Synaptic transmission in an amphibian ganglion. By J. L. Malcolm. *Otago Medical School, Dunedin, New Zealand*

The isolated superior cervical ganglion, with pre- and postganglionic trunks, of the Fijian toad, *Bufo marinus*, survives in an atmosphere of moist oxygen at room temperatures for up to 72 hr. Action potential records show that all fibres synapse; that there are two groups of preganglionic fibres, identified by threshold to electrical stimulation and conduction rates (0·2 and 0·1 m./sec.); that there is no convergence between groups, but each preganglionic axon synapses with, and is capable of initiating a conducted response in, over 90 % of the ganglion cells of its group.

Relatively high concentrations of *d*-tubo curarine, prostigmine or prostigmine and acetyl choline fail to block transmission or produce a synaptic potential. $(K)^+$ and pH changes produce a marked effect on the positive and negative after potentials recorded from the ganglion cells.

Afferent discharges from extraocular muscles. By S. Cooper, P. M. Daniel and D. Whitteridge. *University Laboratory of Physiology, University of Oxford*

Discharges from afferent nerve endings in the inferior oblique muscles of goats have been recorded in single fibres of their branch of the oculomotor nerve. The single sensory units appear to be firing continuously at rates of 18–100 per sec. They respond immediately to any passive increase of tension in the muscle by a higher rate of discharge and they adapt very slowly.

Rapid stretching induces rates of discharge up to 300 per sec. or more. During relaxation, if the resting tension is low, the discharges may fall to a slower rate than the resting rate. During a muscle twitch the sensory unit is completely silent throughout the active contraction, but responds to relaxation as it would to a brief stretch.

The extraocular muscles of goats, like those of man, contain many muscle spindles and it is conjectured that these organs produce the afferent discharges recorded.

The effect of adrenaline on the demarcation potential of mammalian muscle. By G. L. Brown and M. Goffart. *National Institute for Medical Research, London*

We have measured the potential difference between uninjured muscle and an annular burn at the musculo-tendinous junction of the tibialis anterior of anaesthetized cats, using saline-silver chloride electrodes, cathode followers and a galvanometer. Initial values of 30–50 mV. were recorded; the potential difference became stable some 10 mV. lower in 30 min. Arterial injection into the muscle of 10 μg. adrenaline in 0·25 c.c. causes an increase of 5–10 % in demarcation potential which reaches its maximum in 2–5 min. and lasts about 15 min. In extent and time-course it resembles closely the increase in twitch tension which accompanies it. The rise is followed by a depolarization lasting 30 min. or more. Intravenous adrenaline and stimulation of the sympathetic chain also produce these effects.

The contribution of connective tissue impedance to the spatial spread of excitability in the frog's sciatic trunk. By C. Rashbass and W. A. H. Rushton. *Physiological Laboratory, University of Cambridge*

The excitability of this nerve differs in certain respects from the expectations of the simple cable theory, e.g. threshold excitation may arise 3 mm. away from the applied cathode. We believe this to be largely due to the electric impedance of the epineurium upon the following grounds:

(*a*) The discrepancy is hardly detectable in nerves stripped of the connective tissue sheath.

(*b*) There is a large potential drop across the epineurium directly measured during current flow.

(*c*) The observed potential of the interstitial fluid within the connective tissue is exactly that which would give on the simple cable theory the excitability which we obtain.

Membrane potentials in single fibres of the frog's sartorius muscle. By A. L. Hodgkin and W. L. Nastuk. *Physiological Laboratory, University of Cambridge*

Graham & Gerard (1946) and Ling (1948) showed that the potential difference across the surface membrane of a resting muscle fibre could be measured directly with a microelectrode. We have repeated these experiments with equipment capable of recording both action potentials and resting potentials from a microelectrode with an external tip diameter of 0·5 μ. The microelectrodes

were filled with Ringer solution, 0·118M-KCl or 3M-KCl. All three methods gave essentially similar results, but the last method is believed to be the most accurate, since the electrode resistance is relatively low and junction potentials should be small. Using electrodes containing 3M-KCl, sixty-five experiments at 19° C. gave an average resting potential of 87 mV. and an average action potential of 115 mV. At 7° C., corresponding figures for thirty-one experiments were: resting potential 83 mV., and action potential 121 mV.

REFERENCES

Graham, J. & Gerard, R. W. (1946). *J. cell. comp. Physiol.* **28**, 99.
Ling, G. (1948). *Feder. Proc.* Pt. I, **7**, 72.

The 'fundamental' mechanical change in muscle. By A. V. HILL. *Biophysics Research Unit, University College, London*

The tension of a skeletal muscle, rapidly stretched about 10 % of its length shortly after a maximal shock, rises immediately to about that found in a tetanus and remains nearly steady until relaxation occurs. The extensibility of the contractile elements decreases suddenly after the latent period and the quick stretch pulls out the series elastic elements, raising the tension to the maximum which the active elements can bear. In an ordinary twitch relaxation sets in before the contractile elements have had time to stretch the elastic ones enough to give the full tension: once the contractile elements have become active a quick stretch obviates the slow internal shortening and shows up the full tension immediately. The internal change from rest to activity is fully developed within about 2 % of the time normally occupied in a twitch.

REFERENCE

Gasser, H. S. & Hill, A. V. (1924). *Proc. Roy. Soc.* B, **96**, 398.

Some properties of the Pacinian corpuscle. By J. A. B. GRAY and J. L. MALCOLM. *National Institute for Medical Research, London*

Single Pacinian corpuscles (Gray, 1947) were stimulated mechanically by rectangular pulses from a critically damped Rochelle salt crystal. Action potentials were recorded from the corpuscle or its nerve. The corpuscle responds at 'on' and 'off' to displacements of 0·5 μ in 100 μ sec., the threshold for 'on' usually being lower than for 'off'. Corpuscles follow frequencies up to 700/sec. The recovery curves to electrical and mechanical stimuli are similar. The latency of response to a mechanical stimulus decreases from 1·5 msec. at threshold to 0·5 msec. at 5 times threshold, but the latency after an electrical stimulus falls to 0·3 msec. at 1·3 times threshold. A subthreshold mechanical

stimulus facilitates a subthreshold electrical stimulus; the time to peak of the facilitation curve equals the latency to a threshold mechanical stimulus. The facilitation may coincide with a non-propagated potential at the ending. The latency may be due to the natural period of the corpuscle or a spreading of depolarization in the nerve terminals.

REFERENCE

Gray, J. A. B. (1947). *J. Physiol.* **106**, 34 *P*.

Pituitary growth hormone and foetal growth. By J. F. D. Frazer, A. St G. Huggett and F. X. Wohlzogen. *Physiology Department, St Mary's Hospital Medical School, London*

Walton & Hammond (1938) suggest the maternal growth hormones affect foetal growth. This was tested in rats with Young's (1941) crude extract of the anterior pituitary lobe containing largely growth hormone and also some gonadotrophins.

Four groups of rats were injected daily with 0·33 c.c. of Young's extract from (*a*) 10th to 20th day of pregnancy, (*b*) zero (sperms in vagina) to 10th day, (*c*) zero to 20th day, (*d*) pregnant but uninjected. All animals were killed at the 21st day, the normal date of full term in rats.

Daily weighings and foetal weights at the 21st day showed maternal weight deposition was increased in all animals injected. The foetuses in group (*a*) were normal weight; the others were only of 16–17 days' weight and development, presumably due to gonadotrophins delaying implantation. The hormone therefore either fails to pass the placenta or else fails to excite foetal growth.

REFERENCES

Walton, A. & Hammond, J. (1938). *Proc. Roy. Soc.* B, **125**, 311.
Young, F. G. (1941). *Brit. med. J.* (ii), 897.

Failure of nerve homogenates to catalyse the coupled breakdown of acetylcholine and energy-rich phosphate. By A. S. V. Burgen and F. Hobbiger. *Department of Pharmacology, Middlesex Hospital Medical School*

It has been claimed by Rapp (1947) that frog nerve homogenates contain an enzyme that catalyses the coupled breakdown of acetylcholine and creatine phosphate in the presence of a high concentration of eserine (0·2 mM). This reaction might provide an alternative pathway for acetylcholine catabolism and, since synthesis of acetylcholine is known to proceed in the presence of

choline and energy-rich phosphate, also a possible system for regulating the content of acetylcholine in nervous tissue.

Repetition of these experiments, using 1 mg. of homogenized frog or guinea-pig sciatic nerve per ml. substrate as a source of enzyme, has failed to show any coupled breakdown of creatine phosphate and acetylcholine at 10–30° C. (substrate: 1·0 mM acetylcholine bromide; 1·0 mM sodium creatine phosphate; 0·2 mM eserine salicylate in Ringer solution at pH 7·0). Substitution of ATP or ATP plus creatine for the creatine phosphate as an alternative source of energy-rich phosphate was also ineffective.

REFERENCE

Rapp, G. W. (1947). *Arch. biochem.* **12**, 13.

Action of acetylcholine on rabbit auricles in relation to acetylcholine synthesis. By E. Bülbring and J. H. Burn. *Department of Pharmacology, Oxford*

The acetone-dried powder of fresh rabbit auricles, incubated according to the method of Feldberg & Mann (1946), synthesizes acetylcholine in varying amounts, the mean figure being 40 μg/g. powder per hr. The powder from auricles, which have stopped beating after being kept in a bath of Tyrode for 24 hr., synthesizes less acetylcholine, the mean figure being 15 μg./g./hr.

It has previously been shown that small doses of acetylcholine, which depress the beat of a fresh auricle, can start the beat of a stopped auricle; this then continues to beat over a long period. It has now been found that the synthesis of acetylcholine by a powder made from fresh auricles is inhibited by the addition of acetylcholine at the beginning of incubation. On the other hand, the synthesis of acetylcholine by a powder made from stopped auricles is stimulated by the addition of acetylcholine.

REFERENCE

Feldberg, W. & Mann, T. (1946). *J. Physiol.* **104**, 411.

Substances which stimulate the secretion from the cortex of the isolated adrenal. By Marthe Vogt. *Pharmacological Laboratory, University New Buildings, Teviot Place, Edinburgh*

Most substances which enhance the secretion of the adrenal cortex do this indirectly by releasing corticotrophic hormone from the anterior lobe of the pituitary. Two exceptions to this rule have been found, one being KCl and the other adenosine-triphosphate.

Thiosulphate clearance in the cat. By M. GRACE EGGLETON and Y. A. HABIB. *Physiology Department, University College, London*

The clearance of sodium thiosulphate is said to be identical with creatinine clearance in the dog (Gilman, Philips & Koelle, 1946; Pitts & Lotspeich, 1947), and with inulin clearance in man (Newman, Gilman & Philips, 1946). A comparison of its clearance in the cat (nembutal anaesthesia) with that of creatinine, which is itself unaffected by changes in creatinine concentration in the plasma and may therefore be accepted as a measure of Glomerular filtration rate (G.F.R.), indicates that in this species, thiosulphate is actively secreted.

The results of thirty observations of the thiosulphate/creatinine clearance ratio vary, in orderly fashion, from 3·05 at 4·5 mg. thiosulphate/100 c.c plasma to *c.* 1·2 at 100 mg./100 c.c. plasma. Calculation of the amount actively secreted shows that this is directly related to G.F.R. When expressed as mg./min./100 c.c. G.F.R., this value rises steeply with increasing concentration in the plasma until *c.* 30 mg./100 c.c. plasma is reached and thereafter remains steady: i.e. there appears to be a definite *Tm* value for thiosulphate secretion.

REFERENCES

Gilman, A., Philips, F. S. & Koelle, E. S. (1946). *Amer. J. Physiol.* **146**, 348.
Newman, E. V., Gilman, A. & Philips, F. S. (1946). *Johns Hopk. Hosp. Bull.* **79**, 229.
Pitts, R. F. & Lotspeich, W. D. (1947). *Proc. Soc. exp. Biol., N.Y.*, **64**, 224.

The arterial blood pressure responses to adrenaline and noradrenaline in dogs surviving adrenalectomy and sympathectomy. By MARY F. LOCKETT. *Pharmacology, University College, London*

Adrenalectomized dogs, which were maintained in good health by the injection of adrenal cortical extract, showed no alteration in the rises of blood pressure following the intravenous injection of adrenaline and noradrenaline from those of the control period. These same dogs, when maintained on desoxycorticosterone acetate without an accompanying high salt diet, gave an inverted response to adrenaline and a normal response to noradrenaline; this change was reproduced whenever the plasma chlorides were reduced below 500 mg./100 ml., and the plasma sodium was decreasing, when there was also some haemoconcentration and prolongation of the leg to leg circulation time, but no marked rise in the non-protein nitrogen of the blood. Such dogs responded to 5 μg. of noradrenaline and 5 μg. of adrenaline by a change of blood pressure (20–30 mm. Hg).

Sympathectomized-adrenalectomized dogs showed the same alterations in the adrenaline responses when insufficiently treated with DOCA, but were sensitive to 1 μg. or to 2 μg. of either compound.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

15 *January* 1949

The effect of thermal stimuli on the circulation in the human colon.

By John Grayson. *Department of Physiology, University of Bristol*

The object of this demonstration is to show vascular responses in the human large intestine. The subject is a patient with a colostomy performed about nine months ago. Needle thermocouples, inserted into the small knuckle of bowel

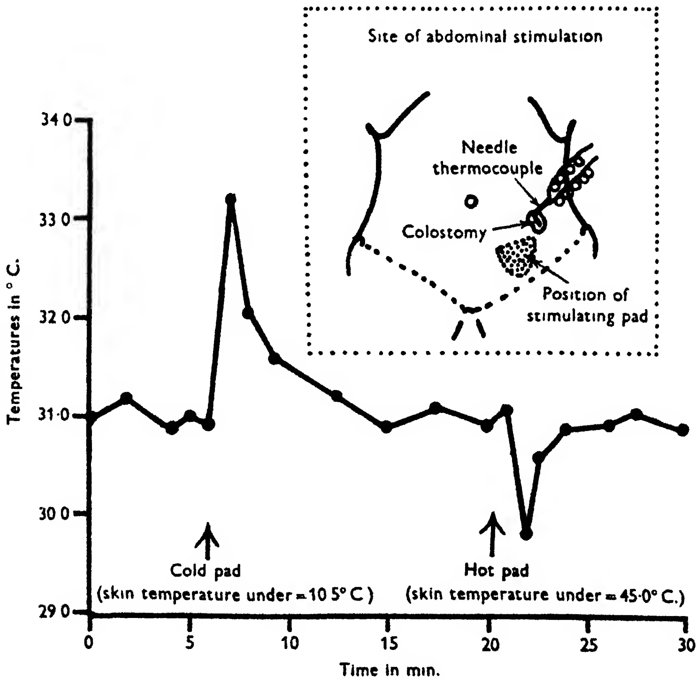


Fig. 1. Reactions of the colonic circulation to heat and cold stimuli applied to the abdominal wall.

exposed on the surface of the abdomen, record temperature changes in response (i) to thermal stimuli applied to the abdominal wall near the colostomy, (ii) to general body heating. Skin temperature changes from an index finger are shown for purposes of comparison.

(i) The effect of thermal stimulation of the abdominal wall in a typical experiment is shown in Fig. 1. A cold, ether-soaked pad applied to the

abdomen in the position shown produced a marked rise in tissue temperature, whereas a hot pad produced a definite fall. The responses could not be obtained by stimulating other parts of the abdominal wall and were abolished by the surface application of novocaine to the colostomy. The site of abdominal stimulation producing maximum effect has been found to vary considerably in different subjects. In cases where sufficient colonic tissue was present to make the experiment practicable, cold applied to the colostomy itself was found to produce a generalized vasodilatation throughout the exposed tissue.

(ii) The effect of general body heating was described by the author in an earlier, oral communication to the Society. It consists of a vasoconstrictor response during the early stages of body heating, followed by a vasodilator response after prolonged body heating. The vasoconstrictor phase accompanies the increase in cutaneous blood flow, whereas the vasodilator phase only appears after the rectal temperature has risen.

Animal decompression chamber and ancillary equipment.

By D. N. Walder. *Department of Physiology, University of Bristol*

This apparatus consists of a cast aluminium rectangular decompression chamber, closed in front by a glass-panelled door. The internal dimensions are $17 \times 11 \times 10$ in. Five separate gas pipe-line entries, two pairs of low-voltage terminals and one pair of high-voltage terminals are built into one side wall of the chamber.

The ancillary equipment is mounted on a control board and includes (1) a barostat, by means of which any predetermined simulated height can be accurately maintained within ± 50 ft., (2) an oxygen flow meter, calibrated to operate at an absolute pressure of 120 mm. Hg, (3) a humidifier to ensure saturation of the oxygen supply, and (4) an oxygen gas analyser for intermittent sampling of the chamber gases.

With the pump and motor used, 40,000 ft. can be attained in 1·3 min.; the maximum altitude attainable is 80,000 ft., reached in 4 min.

Apparatus for making rapid successive measurements of static surface tension by the ring and torsion balance method.

By D. N. Walder. *Department of Physiology, University of Bristol*

This apparatus consists of a torsion balance travelling on rails so that it can be quickly moved into position over any one of a series of vessels containing the liquid under investigation. It is basically the same as that described by du Nouy (1926), but incorporates some refinements.

Torsion is transmitted to the torsion wire of the balance through a reduction gearbox giving a very accurate end-point to determinations. A spring-loaded

clutch between the gear box and torsion wire enables coarse adjustments of torsion and zero adjustment of the apparatus to be performed rapidly. The truck carrying the balance incorporates a brake by means of which the whole apparatus can be kept absolutely rigid during measurements. The ring suspended from the arm of the balance is lowered on to the surface of the liquid by lowering the whole balance assembly on a vertical steel shaft, this operation being controlled by means of a spring-loaded lever system.

REFERENCE

du Nouy, P. Lecomte (1926). *Surface Equilibria of Colloids.* New York: Chemical Catalog Company.

Class apparatus for the perfusion of the isolated mammalian heart.

By A. F. Rogers. *Department of Physiology, University of Bristol*

Oxygenated Ringer-Locke solution is conveyed from a reservoir by a short rubber tube to a vertical glass tube surrounded by a water-jacket. The glass tube ends in a cannula on to which the aorta is tied. Immediately above the cannula are two side tubes, one of which carries a thermometer while the other is sealed by a rubber tube through which drugs can be injected.

The water-jacket has an opening at its upper end by which it can be filled (with distilled water) and another at the lower end for emptying it. There is also a vertical side tube opening into the water-jacket at its upper and lower ends (see Fig. 1). A flame from a micro-burner applied to the side tube causes a current of hot water to rise up the side tube while cold water enters it from below, thus heating the water-jacket throughout by means of a hot-water circulation. The central tube and the water-jacket are made of 'Pyrex' or 'Hysil' glass and are fixed to an old Haldane stand by 'Terry' spring clips. The stand also carries a reservoir (height adjustable), a bar to which the apex of the heart can be sewn, a writing lever, and a water can for use when heating or cooling the pace-maker separately.

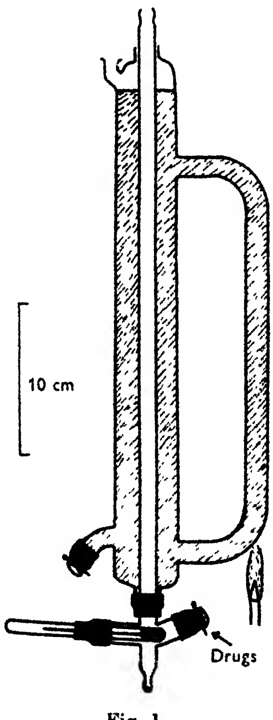


Fig. 1.

The temperature of the issuing saline is controlled by adjusting the flame of the micro-burner, and remains fairly steady provided there are no sudden changes in flow. The apparatus has proved successful in the hands of students over several years.

The effect of synchronizing light and sound stimuli with various components of the electro-encephalogram. By W. G. WALTER and H. W. SHIPTON. *The Burden Neurological Institute, Bristol*

Bright flashes of light and loud sounds evoke potential changes in the cortex which can be detected in human subjects with suitable amplification. These responses are recorded and also passed through circuits which provide frequency selection, squaring, limiting and differentiation; the resulting signals initiate integrating circuits from which pulses are derived to trigger a xenon gas discharge tube and a sound generator at controllable intervals after the original cerebral response. The trigger can also be set to count down and deliver stimuli at a subharmonic frequency at any chosen phase of a rhythmic component, or at any interval after a transient one.

With this device it is possible to distinguish between spontaneous cerebral discharges and those which are evoked or modified by the stimuli. When the stimulus is synchronized with a spontaneous autonomous rhythm the phase and time relations between the two do not affect the record, whereas when the cerebral activity is associated with the afferent signals, adjustment of the frequency selective circuit and phase relations determines whether the feedback is positive or negative with corresponding increment or decrement of the response. The method will be demonstrated on normal subjects, but has been found particularly effective in evoking seizures of various sorts in clinical studies.

Modification of the central action of curare by various agents. By S. SALAMA. *Middlesex Hospital Medical School, London*

In previous communications I reported that intraventricular injections of D-tubocurarine enhances the knee jerk, flexor reflex and the crossed extensor reflex and finally 'spontaneous' convulsions take place.

Intraventricular injection of certain substances produced the following results:

(1) Atropine had a mild inhibitory action on curare convulsions.

(2) Acetylcholine momentarily inhibited the hyper-reflexia produced by curare. This inhibition is slightly reduced (but not annulled) by atropine.

(3) Eserine produced momentary inhibition.

(4) Hexaethyltetraphosphate had a similar action to that of eserine; it also produced a rise of blood pressure by a central action.

(5) Potassium chloride and tetramethyl ammonium iodide inhibited curare convulsions.

The rate of discharge of the extra-ocular motoneurones. By
G. Reid and D. Whitteridge. *Department of Physiology, University of Melbourne, and University Laboratory of Physiology, Oxford*

Although the duration of twitch contractions of the extra-ocular muscles of goats and cats is known to be short and their fusion frequency to reach 350/sec. (Cooper & Eccles, 1930), the rate of discharge of the motor units in reflex activity has apparently not been recorded. Records were obtained from single-fibre preparations of the nerve to the inferior oblique muscle of cats, and also from muscle units by means of fine wire electrodes inserted into various muscles. Galvanic stimulation of the labyrinth resulted in rates of discharge up to 60/sec., but much higher rates were obtained during sudden flexion or rotation of the head on the neck. In single units the rate of discharge reached 120–170/sec. during the movement, and subsided to steady rates of 5–75 impulses/sec. during maintained flexion. In records from several units simultaneously, grouping of impulses sometimes occurred during the movement of the head.

REFERENCE

Cooper, S. & Eccles, J. C. (1930). *J. Physiol.* **69**, 377.

Factors affecting the static surface tension of serum.
By D. N. Walder. *Department of Physiology, University of Bristol*

In a previous communication (Walder, 1948) a relationship was shown between the static surface tension of the blood serum and the susceptibility to decompression sickness in man. It is now shown: (1) That the static surface tension is inversely related to the total protein concentration of the serum as estimated by the copper sulphate method (Phillips, Van Slyke, Dale, Emerson, Hamilton & Archibald, 1944) such that $r = -0{\cdot}46$ and $P = <0{\cdot}001$ (51 observations) and by the biuret method $r = -0{\cdot}86$ and $P = <0{\cdot}001$ (26 observations). But (2) it is not related to the total cholesterol concentration as estimated by a modified Meyer's method (Leiboff, 1924), $r = 0{\cdot}21$ and $P = >0{\cdot}1$ (30 observations). (3) Further, the total protein concentration is inversely related to the individual's normal daily fluid intake, $r = -0{\cdot}92$ and $P = <0{\cdot}001$ (31 observations). These findings would explain the work of Thompson, Stewart, Warwick, Bateman, Milne & Gray (1944), who found that subjects with a high fluid intake were more resistant to decompression sickness than those with a low fluid intake.

REFERENCES

Leiboff, S. L. (1924). *J. biol. Chem.* **61**, 177.
Phillips, R. A., Van Slyke, D. D., Dale, V. P., Emerson, K., Hamilton, P. B. & Archibald, R. M. (1944). *Bull. Army Med. Dep.* **71**, 66.
Thompson, J. W., Stewart, C. B., Warwick, O. H., Bateman, G. L., Milne, D. J. & Gray, D. E. (1944). Committee on Aviation Medicine, *Report* No. F.P.M.S. D-3, National Research Council.
Walder, D. N. (1948). *J. Physiol.* **107**, 43 *P*.

Nodes of Ranvier in the central nervous system.

By A. Hess and J. Z. Young

There has been doubt about the existence of nodes in the central nervous system because the myelin of central nerve fibres is readily altered in preparation. Methylene blue, however, provides reliable evidence that the fibres are not equally permeable at all points, which is a more interesting way of saying that they have nodes of Ranvier. Ranvier himself denied this, but they were reported by Tourneux & Le Goff (1875) and by Cajal (1899), more recently by Feindel, Allison & Weddell (1948) and by Huxley (1948). Fibres of the spinal cord of the rabbit teased after staining by intravascular injection with methylene blue show them well, at intervals which, as in peripheral nerve, increase with the fibre diameter. Staining such preparations with osmium tetroxide shows that the myelin sheath is interrupted at these points of greater permeability, but so far the difficulties produced by the flowing of the myelin have prevented detailed comparison with peripheral nerve.

REFERENCES

Cajal, S. R. (1899). *Textura del Sistema Nervioso del Hombre y de los Vertebrados.* Madrid: Moya.

Feindel, W. H., Allison, A. C. & Weddell, G. (1948). *J. Neurol., Neurosurg., Psychiat.*, **11**, 227.

Huxley, A. F. (1948). Physiol. Soc. Meeting (unpublished), 18 December.

Tourneux, F. & Le Goff, R. (1875). *J. de l'Anat. et de Physiol.* 403.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

19 *February* 1949

A technique for investigating the properties of a single mammalian sensory ending.

By J. A. B. GRAY and J. L. MALCOLM. *National Institute for Medical Research, Hampstead, London*

The isolated Pacinian corpuscle preparation has been described previously (Gray, 1947). The technique has been modified to allow both mechanical and electrical stimulation of the corpuscle in the intact animal with the preparation immersed in liquid paraffin. The recording circuits and the means of electrical stimulation need no description.

The mechanical stimuli are transmitted to the corpuscle through a glass stylus from a Rochelle salt crystal. This crystal (Rothermel type 4D41), 15 mm. square, is mounted in a brass case on three of its corners. The remaining free corner is attached to the upper end of the glass stylus. A potential difference of up to 50 V., from a source having an output impedance of 1000 Ω, is applied across the crystal and causes it to bend so that the free corner has a maximum vertical movement of about 25 μ. Damping is necessary and is provided by an oil film, which fills a 0·5 mm. gap between the free corner and a platform placed behind it.

The form of the force-time curve developed by the crystal, when a rectangular voltage pulse is applied, has been recorded as the voltage generated in another, identical crystal against which the stylus of the first was placed. Such records show a rising time of 100 200 μsec., depending on the crystal and degree of damping.

The brass case housing the crystal is mounted on a universal clamp; a fine vertical adjustment is provided. The whole is clamped to the support of the stage on which the preparation is fixed, and the structure between the two is kept as rigid as possible.

Some results obtained with this technique have already been published (Gray & Malcolm, 1948).

REFERENCES

Gray, J. A. B. (1947). *J. Physiol.* **106**, 34 *P*.
Gray, J. A. B. & Malcolm, J. L. (1948). *J. Physiol.*, Proc. (in the Press).

A convenient nerve-muscle preparation from the gracilis of the cat.

By G. L. Brown and B. D. Burns. *National Institute for Medical Research. Hampstead*

For experiments on neuromuscular transmission we sought a preparation which would enable us to stimulate the nerve-free end of a muscle and record the action potentials so produced. This demanded a small muscle with long, parallel fibres and having a blood supply so arranged that a reasonable length of muscle could be dissected free. A preparation like this has been made from the gracilis muscle of the cat. The gracilis is a wide, flat muscular sheet arising from beneath the symphysis pubis; it is inserted into the tibia by a thin aponeurosis. Its nerve supply enters the lateral edge of the muscle about 1 cm. distal to its origin.

Our preparation is made from the distal end of the medial margin of the muscle. In decerebrate cats, the leg is immobilized by section of the sciatic nerve at its exit from the sciatic notch and of the femoral nerve where it leaves the abdomen. The incision for the femoral nerve is then continued distally along the middle of the medial aspect of the thigh, over the knee joint to the middle of the calf. The skin is freed from the underlying muscle, and the medial edge of the gracilis is defined and separated with a sharp knife from the underlying semi-membranosus and semi-tendinosus. The gracilis can now be raised with a spatula and its under-surface inspected. An artery and vein are seen to run from the underlying muscles into the substance of the gracilis, entering it some 6 mm. from its medial edge. The under-surface of the muscle can now be illuminated with a small bulb, and the blood vessels can be seen through the muscle as they ramify towards the insertion. A sharp knife is now thrust through the tendon near the insertion and drawn along the edge of the muscle parallel to the fibres, so as to separate a strip, roughly triangular in shape, some 2 mm. wide at the tendon and 0·5–1 cm. in width, where it ends near the middle of the muscle. The dimensions of the strip are determined by the size of the cat and the position of the blood vessels, since care is taken to avoid cutting visible branches, and the cut is so arranged that a major arterial branch runs along its length; strips containing a length of some 5 cm. of muscle can sometimes be obtained, and it is seldom that a length of 3 cm. cannot be freed.

In some cats the arrangement of the arteries at the end of the muscle is such that a satisfactory strip cannot be cut from the medial edge. In these circumstances it is often possible to cut a strip from the adjacent muscle more laterally.

For recording, the leg is immobilized through drills in the distal ends of the tibia and femur and another in the great trochanter of the femur. A thread is tied to the tendon of the strip and sufficient traction is applied to separate the strip from the rest of the muscle. The skin of the thigh is sewn to a flexible

metal ring, and the bath so formed is filled with liquid paraffin which is kept at 38° C. by radiant heat. Fine platinum wire electrodes are applied to the nerve and to the tendinous end of the muscle for stimulation, and records of the muscle potentials are led off by two platinum wires laid transversely on the surface of the strip.

It is easy to plot electrically the anatomical arrangement of nerve endings and muscle fibres in this preparation. If the nerve is stimulated and the recording electrodes (3 mm. apart) are laid on the musculo-tendinous junction, a monophasic action potential is observed. Movement centrally of the electrodes by about 0·5 cm. gives a completely diphasic potential; a further central movement of the same distance makes the potential become complex and irregular; yet another advance of 0·5 cm. yields a simple diphasic potential, but of opposite sign to the first.

These observations suggested that the muscle fibres received their nerve supply at, or about the place where the potential became complex. Administration of curarine showed that this was true, since at this point large end-plate potentials are readily recorded.

The close circumscription and accessibility of the end-plate region in this strip makes it useful for direct stimulation when uncurarized. By appropriate placing of the electrodes it is possible to record clear diphasic responses to direct excitation which are of opposite sign to those evoked by nerve stimulation, and render the muscle refractory to excitation arising from the end-plate region.

The properties of polymethylene bistrimethylammonium salts.

By W. D. M. Paton and E. J. Zaimis. *National Institute for Medical Research, Hampstead*

The properties of the series of polymethylene α-ω-*bis*trimethylammonium salts ($N^{+}(CH_3)_3.(CH_2)_n.N^{+}(CH_3)_3.2I''$) are best demonstrated by the actions of the decane (C10) and pentane (C5) derivatives, which respectively paralyse transmission at the neuromuscular junction and at the ganglionic synapse. Adjacent members of the series have similar properties in less degree. A brief report of some of these actions has already been made (Paton & Zaimis, 1948*a*, *b*).

C10 is highly potent in causing neuromuscular block. A dose of 30–40 μg./kg. injected intravenously into the cat anaesthetized with chloralose, or 2 μg./kg. given by close arterial injection, usually paralyses tibialis completely to excitation through its motor nerve. During such paralysis direct electrical stimulation of the muscle is still effective, and the action potentials of the motor nerve are unchanged. If a tetanus is applied to the nerve during a partial paralysis of the muscle by C10, the tension developed during the tetanus is well-sustained. After the tetanus, the tension of single twitches is neither depressed (as is observed

in the presence of eserine) nor enhanced (as with a paralysis due to *d*-tubo-curarine chloride). A striking feature of the action of C10 is the sparing of the respiratory muscles relative to those of the leg.

Anticholinesterases have little influence on the curarizing action of C10; they may, indeed, slightly increase its effect. But C5 in a dose 10–100 times greater than that of C10 is an efficient antagonist in all species investigated; it will both reverse the paralysis due to C10 given before it and diminish the effect of C10 given after it. Previous administration of *d*-tubocurarine chloride, or of compounds related to it, also antagonizes C10, although *d*-tubocurarine chloride maintains its activity when injected after C10. C10 varies greatly in its potency with different species, being very active in cat and in man (Organe, Paton & Zaimis, 1949), and progressively less active in rabbit, mouse and rat. The ratio of the dose of C10 in the rat to that equally effective in the cat is about 100; for *d*-tubocurarine chloride, the ratio is about 0·5.

C10 is a powerful stimulant of the frog's rectus abdominis, and will elicit a contraction in a concentration of $1\text{–}2 \times 10^{-6}$. This strong stimulant action on skeletal muscle is not seen after intravenous injections into the cat, although fine fasciculations of tibialis, and occasionally a feeble contraction lasting a few seconds, may appear. After rapid intra-arterial injection, however, during the intermission of stimulation through the nerve, a vigorous twitch precedes the paralysis. It is characteristic, too, that before the onset of paralysis of the response of the muscle to maximal nerve shocks, the twitch tension is increased for a short time, due to repetitive firing by the muscle fibres. C10 possesses some anticholinesterase action, which may account for some of the effects just mentioned. It is considerably more active against 'true' than 'pseudo'-cholinesterase. It has a very feeble muscarine-like action, and no detectable atropine-like action. In large doses, it paralyses autonomic ganglia.

C5, although virtually devoid of curarizing action at the neuromuscular junction, is very active in paralysing transmission at the ganglionic synapse. A small dose (0·2 mg./kg.) injected intravenously into the cat anaesthetized with chloralose causes relaxation of the nictitating membrane (excited to contraction by preganglionic stimulation). A slightly larger dose causes a fall of blood-pressure, which is abolished by a paralysing dose of nicotine. A further increase in dose prevents the bradycardia due to peripheral stimulation of the cut vagus. Tested by the technique of Feldberg & Lin (1948), C5 in small amounts abolishes the peristaltic reflex of rabbit intestine. Doses of the drug, however, sufficient to cause paralysis of ganglionic transmission, do not diminish the release of acetylcholine by the preganglionic nerve terminals of the perfused superior cervical ganglion, and do not alter the effects of postganglionic stimulation.

Besides opposing the curarizing action of C10, C5 antagonizes the power of C10 and of acetylcholine to cause contraction of the frog's rectus. C5 possesses

no anticholinesterase activity or muscarine-like action, nor any of the stimulating properties possessed by nicotine.

REFERENCES

Feldberg, W. & Lin, C. Y. (1948). *J. Physiol.* **107**, 37*P*.
Organe, G., Paton, W. D. M. & Zaimis, E. J. (1949). *Lancet*, **256**, 21.
Paton, W. D. M. & Zaimis, E. J. (1948*a*). *Nature, Lond.*, **161**, 718.
Paton, W. D. M. & Zaimis, E. J. (1948*b*). *Nature, Lond.*, **162**, 810.

A respiration recorder. By W. D. M. Paton. *National Institute for Medical Research, Hampstead*

The apparatus provides a compact instrument for use with cats; it records on the smoked drum, the height of the record above the base-line being directly proportional to the respiratory minute volume; it does not need repeated calibration; and it offers little resistance to respiration. The principle involved

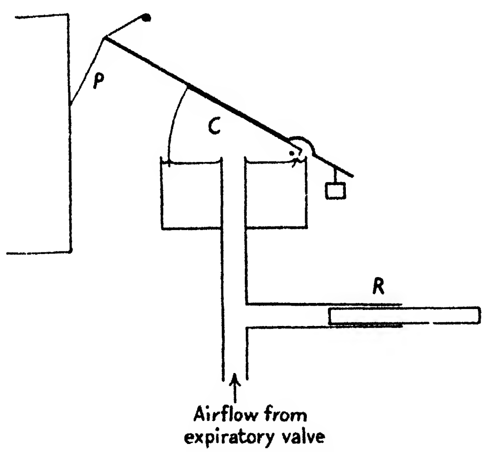
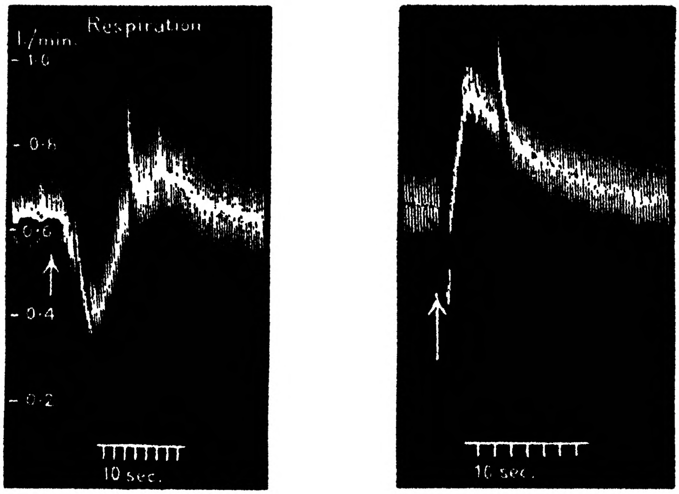


Fig. 1. Diagram of recorder.

is simply explained by an electrical analogy. If charge flows continuously into a capacity, from which it can leak away through a resistance, the potential in the capacity rises exponentially to a limiting value which is a measure of the rate of flow of charge. If the flow of charge is intermittent, the system acts as a smoothing device. Here the flow of 'charge' is the expiratory minute volume of the animal (V l./min.). The 'capacity' is a float recorder (C) (Fig. 1); this also records directly the 'potential' or pressure within it by the rigidly attached frontal writing lever, since the pressure is proportional to the height (P cm.) of the tracing. The 'resistance' (R) is a variable leak consisting of a brass

rod sliding within a glass tube. Certain constructional details require mention:

(1) the *float recorder* is made of Perspex 2 mm. thick, cross-section $6{\cdot}8 \times 5{\cdot}8$ cm. and 7 cm. deep at its widest end. This end is covered with a layer of paraffin wax 15 mm. thick to give it additional weight. It is counter-weighted so that with no air flow the recorder is near the bottom of its excursion. The aluminium writing lever terminates 18·5 cm. from the pivot in a writing point 63 mm. long. (The dimensions given are not critical.)



(*a*) (*b*)

Fig. 2. Cat, 2·8 kg., chloralose. Effect of intravenous injections of (*a*) 200 μg. adrenaline; (*b*) 0·5 mg. lobeline hydrochloride.

(2) The *variable leak* consists of a brass rod 6·34 mm. diameter in a glass tube of inside diameter 6·88 mm. There is appreciable play of the rod in the tube, and a centring device is necessary; a thin brass triangle making three-point contact on the inside of the tube is screwed to the end of the rod in the tube, and a bearing attached to the tube carries the other end. If the rod or the tube is not quite uniform in diameter, they may be marked so that the alinement at the time of calibration is maintained.

Calibration. The deflexion of the float-recorder varied linearly both with the pressure inside it and with the volume of air entering it (1·49 mm. water and 25·8 c.c./cm. deflexion respectively). The resistance also proved to be satisfactorily laminar for air flows up to 1 l./min., and it was directly proportional to the length of overlap (*d* cm.) of the rod and the tube. The final calibration of airflow against deflexion on the drum was also linear with an error less than 5 %. From these figures the apparatus can be characterized by the equation for

the steady state, $V = \frac{0{\cdot}371\,P}{d+0{\cdot}5}$, and by the half-time of the system (which depends on the capacity and resistance) which equals 2·93d sec. The constants do not change with time, and the calibration remains constant indefinitely. The distortion of the time scale of respiratory events by the damping action is rarely significant.

Use. It is essential that valves used should be leak-free and junctions air-tight; moistened rubber valves of low resistance in a Perspex holder are suitable. A length of tubing at least 0·5 m. long between valves and apparatus prevents condensation in the resistance.

Since respiration is intermittent, the tracing has an appreciable width. This is, in fact, an approximate measure of the tidal air, which may be determined more accurately from the minute volume and the respiratory frequency. For most purposes it is sufficiently accurate, when ascertaining the minute volume, to measure the height from the base-line to the middle of the tracing, since the rise and fall of the recorder are nearly linear. The resistance is made variable so that a convenient deflexion can be obtained for a given minute volume. The resistance offered to the cat's respiration is usually $\frac{1}{2}$–$\frac{3}{4}$ cm. water and need never exceed 1 cm. water.

Fig. 2*a* and *b* illustrates the use of the recorder, showing apnoea and compensatory hyperpnoea due to adrenaline, and respiratory stimulation by lobeline.

I am greatly indebted to Mr R. C. Blakemore and Mr G. Pierson for the construction of the recorder and the valve-holder.

The effect of carbon dioxide on perfused lungs. By HELEN DUKE.
Department of Physiology, Edinburgh University

Isolated lungs of cats and dogs have been perfused with heparinized blood through the pulmonary artery at constant-volume inflow. The lungs have been ventilated by positive or negative pressure. In the dog, changes in the ventilating gas mixture from atmospheric air to air containing 5–10% CO_2 have produced a diminution of the volume of blood in the lungs with or without a slight rise of pulmonary arterial pressure. The volume of blood in the lungs shows an inverse relationship to the concentration of CO_2 in the arterial blood. In cats' lungs the same concentrations of CO_2 have a marked pressor effect. Analogous findings have been reported by Euler & Liljestrand (1946) in the whole anaesthetized cat.

REFERENCES

Euler, U. S. v. & Liljestrand, G. (1946). *Acta Physiol. Scand.* 12, 301.